1-2-2013

Thick Ascending Limb Nitric Oxide Production And Inhibition Of Nkcc2 Activity Are Impaired In Angiotensin Ii-Induced Hypertension

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THICK ASCENDING LIMB NITRIC OXIDE PRODUCTION AND INHIBITION OF NKCC2 ACTIVITY ARE IMPAIRED IN ANGIOTENSIN II-INDUCED HYPERTENSION

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2013

MAJOR: PHYSIOLOGY

Approved by:

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Advisor Date

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DEDICATION

I would like to dedicate this dissertation to my mom whose encouragement and example of perseverance bolstered my desire to pursue a career in science; to my dad for supporting me and understanding the decisions I have made throughout my life; to my sisters Yanina and Jennifer who I missed tremendously all these years; to my friends that accompanied me through this process and to my sweetheart André for bringing so much love and joy to my life.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my mentor Dr. Garvin for his guidance and teachings.

Thanks to the members of the committee Dr. Rossi, Dr. Yingst, Dr. DiCarlo and Dr. Lash.

I would like to thank Dr. Garvin's lab members and to the members of Hypertension and Vascular Research in particular Dr. Pablo Ortiz and Dr. William Beierwaltes.

I would like to thank for all the administrative help to Chris Cupps and Emmett Bowen.

I would like to thank all my friends for their love and support.

Finally, I would like to thank the Interdisciplinary Biomedical Sciences Program from Wayne State University and the American Heart Association for their financial support (AHA11PRE7510005).

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CHAPTER 1

GENERAL BACKGROUND

1. a. Introduction to hypertension

Cardiovascular disease is the leading single cause of death in the US[1]. One of the most important risk factors for cardiovascular disease is hypertension which is present in about 30% of the U.S. population[2]. Although great advances have been made in the treatment of high blood pressure, about 20% of hypertensive people do not respond to treatment[3]. Thus, it is important to continue to investigate the mechanisms that mediate hypertension.

The kidneys play an important role in regulating blood pressure by adjusting Na and water reabsorption and thus affecting extracellular fluid volume. Guyton showed that increases in blood pressure result in increases in Na and water excretion by the kidney by a mechanism called pressure-natriuresis. When the kidney cannot properly eliminate an excess of Na, extracellular body fluid augments and so does blood pressure, therefore inducing natriuresis and returning blood pressure to basal levels. Thus, Guyton established that sustained elevations in blood pressure are the consequence of an impaired ability of the kidney to excrete an excess of Na and water and concluded that hypertension can only occur in the presence of impaired pressure-natriuresis[4]. Therefore, studying the mechanisms that contribute to inappropriately enhanced NaCl reabsorption by the kidney during hypertension could help elucidate new targets for the treatment of high blood pressure.

1. b. Thick ascending limb transport

The thick ascending limb of the loop of Henle (THAL) is a nephron segment that has been shown to participate in blood pressure regulation[5]. It reabsorbs 20 to 30 % of the filtered NaCl load and is characterized by its ability to transport NaCl independently of water, thus generating transepithelial NaCl gradients of up to 120 mEq/L and contributing to the cortico-medullary osmotic gradient[6]. The THAL's ability to dilute intratubular fluid is not only important in NaCl reabsorption but also in water reabsorption. By generating hypotonic tubular fluid, the THAL allows for vasopressin-stimulated water reabsorption by the distal tubule and the cortical collecting duct to take place. Given that the reabsorptive capability of the downstream portions of the nephron is limited, inhibition of THAL's NaCl transport results in profuse diuresis, natriuresis and in reduction of blood pressure as seen after treatment with loop diuretics such as furosemide.

NaCl reabsorption by the THAL occurs *via* transcellular and paracellular pathways (Figure 1); however, transcellular reabsorption drives paracellular ion transport and thus inhibition of the former inevitably results in blockade of the latter[7]. In the THAL, Na entry across the luminal membrane is carried out by 2 main transporters: Na/K/2Cl cotransporter type 2 (NKCC2)[8] and Na/H exchanger (NHE3)[9]. NKCC2 activity accounts for 80% and NHE3 20%of transcellular Na transport. Na exits the cell *via* the Na/K ATPase pump. Cl is transported out by the basolateral Cl channels (in particular ClC NKB) [10] and K/Cl cotransporters (mainly KCC4)[11]. Finally, K exits through both luminal and basolateral K channels (including the renal outer medullary K channel or ROMK) [12-15] and through the basolateral K/Cl cotransporters. K recycling to the lumen is not only important for NKCC2 to function, but also contributes to generation of a lumen positive transepithelial potential which generates the driving force for the

paracellular transport of cations including Na, Ca and Mg.

The role the THAL plays in regulating blood pressure is underscored by the effectiveness of furosemide in decreasing blood pressure[16], by the hypotension observed in Bartter syndrome[17] where THAL NaCl transport is impaired, and by the enhanced THAL NaCl reabsorption observed in salt-sensitive hypertension[5,18-20]. In Dahl-salt sensitive rats, a model of salt-sensitve hypertension, Cl reabsorption by the THAL is enhanced[18-20] and decreases in NO-mediated inhibition of NaCl reabsorption[21], increases in superoxide formation[22] and elevation in NKCC2 levels[23] have been shown to play a role. Therefore, studying the signaling mechanisms that affect NaCl reabsorption by the THAL is important in understanding blood pressure regulation.

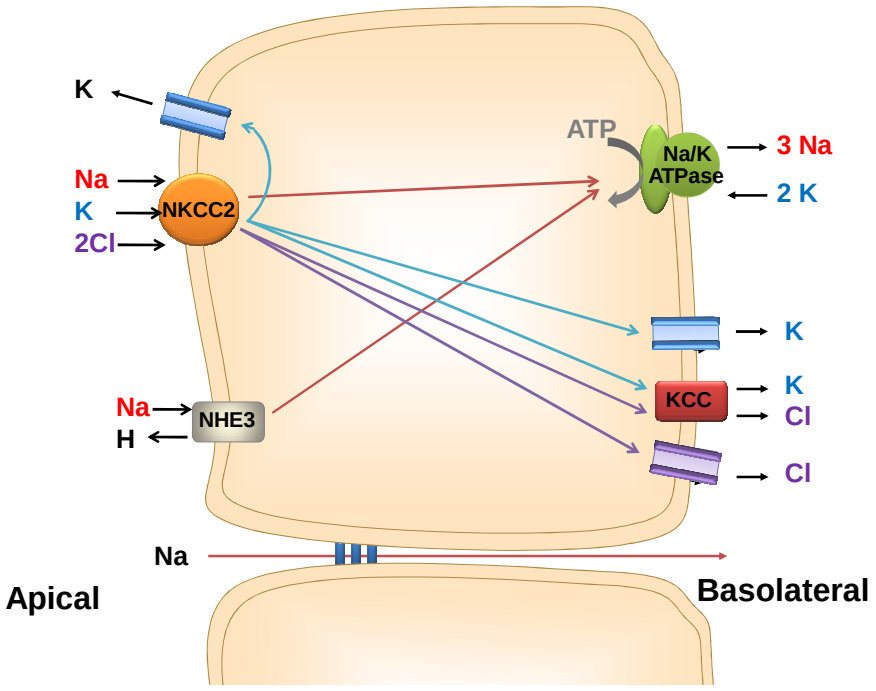

Figure 1. Overview of THAL ion transport. Schematic representation of THAL cells and ion transporters, see explanation in the text.

1. c. Angiotensin II and NO: two important regulators of blood pressure and renal function

Angiotensin II (Ang II) is a key regulator of blood pressure through its actions on the kidneys[24-28], blood vessels[29], heart[30] and central nervous system[31]. Most of its pro-hypertensive actions are mediated by Ang II receptor type 1 (AT1)[32]. The role of Ang II in blood pressure regulation is underscored by the broad utilization of renin inhibitors[33,34], angiotensin I converting enzyme blockers[35,36] and AT1 receptor antagonists[37,38] in the treatment of human hypertension.

Chronic infusion of Ang II in rodents has been extensively used as a model of hypertension[39-42]. In this model, the kidneys play an important role as shown by the decreased elevation in blood pressure observed in WT mice transplanted with kidneys from AT1 knockout mice[24]. Although many studies have explored the contribution of different renal structures in Ang II-induced hypertension[40,43-46], the role of THALs in this model is still not completely understood.

Acutely, Ang II has a biphasic effect on THAL ion transport: at concentrations of 1 pmol/L or lower Ang II inhibits whereas at 1 µmol/L Ang II stimulates NKCC2 activity[47]. Interestingly, Ang II levels in the kidney are in the nanomolar range and they increase further in response to chronic Ang II infusion[48] suggesting that NKCC2 activity is likely to be increased during hypertension. Ang II also stimulates the 10-ps Cl channel in THALs[49] as well as increases superoxide production and protein kinase C (PKC) activity[50], which stimulate NKCC2 activity and NaCl reabsorption by the THAL[51-53]. Finally, chronic infusion of Ang II increases transport-related oxygen consumption, an indicator of NaCl reabsorption by the THAL[54]. Altogether, these data indicate that acutely and chronically Ang II stimulates ion transport by the THAL. However, whether

this is due to direct effects on the transporters or whether Ang II impairs the ability of the THAL to properly produce and respond to natriuretic mediators is not known.

In contrast to Ang II, NO is an endogenous vasodilator and natriuretic factor[55-59]. In the renal vasculature NO induces vasodilatation, increases glomerular filtration rate and elevates renal blood flow[60-62] . In the nephrons, NO inhibits NaCl reabsorption by proximal tubules[63,64], THAL [65-67] and collecting ducts[68]. The importance of endogenously produced NO in maintaining normal blood pressure has been demonstrated by blockade of renal NO synthases (NOS) using low doses of a NOS inhibitor which induced Na retention and salt-sensitive hypertension[69]. In addition, it has been shown that in Dahl salt-sensitive hypertensive rats, renal medullary NO levels and actions in response to physiological stimuli are impaired[70] and oral or intramedullary administration of L-arginine (NOS substrate), increases natriuresis and decreases blood pressure[71-73]. Therefore, Ang II-induced hypertension could also increase Na reabsorption by reducing NO production or impairing NO actions in the kidney. However, findings in this model have been less conclusive and increases in NO production have been found in the renal cortex[74,75].

NO is produced by three NOS isoforms: NOS1 or neuronal, NOS2 or inducible and NOS3 or endothelial. The THAL expresses all 3 NOS isoforms[76]; however, stimuli shown to inhibit THAL NaCl transport such as endothelin-1 (ET-1), L-arginine and clonidine mediate their effects *via* NOS3-mediated NO production[77-79]. These data indicate that NOS3 is the main source of NO responsible for the inhibition of NaCl transport by the THAL.

Chronic increases in Ang II could decrease NO production by the THAL by reducing NOS3 expression and NOS3 phosphorylation at stimulatory sites. In

endothelial cells, chronic exposure to NO reduces NOS3 expression and activity in a negative-feedback fashion[80,81]. Since our lab has shown that acutely, Ang II enhances NO production by the THAL[82] it is possible that the same negative feedback is present in THALs. However, whether chronic elevations in Ang II reduce THAL NOS3 expression *via* NO is not known.

NO inhibits THAL NaCl reabsorption and NKCC2 activity by increasing cGMP[67]. However, cGMP levels are also subject to degradation by phosphodiesterases (PDE) like PDE5[83]. PDE5 limits NO actions in smooth muscle cells by reducing cGMP levels [84,85] whereas Ang II increases PDE5 expression and activity[86]. Interestingly, in Dahl salt-sensitive hypertensive rats, NO-induced decreases in THAL NaCl reabsorption are impaired whereas cGMP production is not affected[21], suggesting that enhanced cGMP degradation could be present in hypertension. However, whether PDE5 is expressed in the THAL and whether enhanced PDE5 blunts NO-induced inhibition of NKCC2 activity in Ang II hypertension are not known.

1. d. Project Aims

We hypothesized that THAL NO production and inhibition of NKCC2 activity are impaired in Ang II-induced hypertension.

In order to test our hypothesis we divided our work into three aims:

Aim I hypothesis: NO production in response to physiological stimuli is impaired in THALs from Ang II-induced hypertensive rats. We measured NO production in response to physiological stimuli as well as NOS3 expression and NOS3 phosphorylation in THALs from vehicle- and Ang II-treated rats.

Aim II hypothesis: Ang II decreases NOS3 expression *via* NO in THAL primary

cultures. We measured NOS3 expression in response to chronic exposure to Ang II. We delineated the signaling pathway by which Ang II reduces NOS3 expression in THALs.

Aim III hypothesis: PDE5 blunts NO-induced increases in cGMP and inhibition of NKCC2 activity in THALs from Ang II-hypertensive rats. We measured cGMP and NKCC2 activity in response to NO in the presence and absence of the PDE5 inhibitor.

CHAPTER 2

NITRIC OXIDE PRODUCTION IN RESPONSE TO PHYSIOLOGICAL STIMULI IS IMPAIRED IN THICK ASCENDING LIMBS FROM ANGIOTENSIN II-HYPERTENSIVE RATS

2. a. Introduction

In the THAL, physiological stimuli such as ET-1[77,87] and L-arginine[65,78] inhibit NaCl reabsorption *via* NO produced by NOS3. The ability of the THAL to produce NO is essential for those stimuli to inhibit NaCl transport, since such a response is absent in THALs from NOS3 KO mice. We have shown that in THALs decreases in NO production in response to physiological stimuli are correlated with decreases in NOS3 expression[88]. Therefore, studying THAL NOS3 expression in pathological states is important to understand the mechanism by which NO production is impaired.

In the THAL increases in NO production in response to most physiological stimuli are mediated by phosphatidyl inositol 3,4,5 tri-phosphate (PIP3) *via* activation of phosphatidyl inositol 3 kinase (PI3K)[77,82,89,90]. Increases in PIP3 enhance NOS3 phosphorylation at serine 1177 elevating NO production whereas blockade of PI3K prevents stimuli-induced NO production. These data indicate that phosphorylation of serine 1177 is an important event in the activation of NOS3 and that defects in this process could lead to impaired NO production.

Our lab has shown that in Ang II-induced hypertension THAL transport is enhanced[54] suggesting that production of inhibitory molecules such as NO could be decreased. Furthermore, cytokines that are increased in response to Ang II reduce NOS3 expression by the THAL[88]. Therefore, we hypothesized that in Ang II-induced hypertension THAL NO production in response to physiological stimuli is impaired and

this correlates with reduced NOS3 expression and decreased phosphorylation at serine 1177.

2. b. Materials and Methods

Drugs: Unless otherwise stated, reagents and drugs were purchased from Sigma (St. Luis, MO).

Ang II-induced hypertension: All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Hospital and Wayne State University. Male Sprague Dawley rats weighing 90-120 g were fed standard chow (0.4% Na) and infused with either vehicle (0.01 N acetic acid) or Ang II at 200 ng/Kg/min *via* osmotic minipumps (Durect; model 1007D) for 5 days (minipump implantation was performed under isofluorane 1.5% in oxygen 0.5 L/min). Mean arterial blood pressure was measured *via* the femoral artery at day 5 after treatment in anesthetized rats (ketamine 90 mg/Kg, xylazine 10 mg/kg).

THAL suspensions: Suspensions were performed as we have done before [50,88,91]. Briefly, the abdominal cavity was opened, and the kidneys flushed with 40 mL ice-cold 0.1% collagenase (Sigma; St. Louis, MO) containing 100 U heparin in physiological saline *via* retrograde perfusion of the aorta. Kidneys were removed and coronal slices cut. The inner stripe of the outer medulla was minced and digested in 0.1% collagenase at 37℃ for 30 min. During each 5 min period, the tissue was gently agitated and gassed with 100% oxygen. Tissue was centrifuged at 60 x g for 2 min; the pellet was re-suspended in cold physiological saline and stirred on ice for 30 min. The suspension was filtered through a 250 μm nylon mesh and centrifuged at 60 x g for 2 min. The pellet was washed and centrifuged again.

Western blot: Protein expression levels were measured using a modification of

our previous technique[88,91]. Tubules were lysed in 500 µL of a solution containing 20 mmol/L HEPES (pH 7.5), 2 mmol/L EDTA, 0.3 mol/L sucrose, 1.0% Igepal CA-630, 0.1% sodium dodecyl sulfate, 5 µg/mL antipain, 10 µg/mL aprotinin, 5 µg/mL leupeptin, 4 mmol/L benzamidine, 5 µg/mL chymostatin, 5 µg/mL pepstatin A, and 0.116 mol/L pf-block (Sigma; St Louis, MO). Debris was removed by centrifugation for 5 min at 5,600xg. Protein concentration was determined by a colorimetric assay (Coomassie Plus protein assay, Pierce, Rockford, IL).

Equal amounts of protein from freshly prepared protein lysates from THAL suspensions were loaded per lane. THAL proteins from one vehicle-treated rat and one Ang II-treated rat were loaded per gel, so every gel had its own control.

NOS3: proteins were loaded in a 6 % SDS-polyacrylamide gel, separated by electrophoresis, and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated in blocking buffer containing 20 mmol/L Tris, 137 mmol/L NaCl, 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk for 60 min and then with a 1:1,000 dilution of an NOS3-specific monoclonal antibody (BD Transduction Laboratories, San Diego, CA) in blocking buffer for one hour at room temperature. The membrane was washed with TBS-T and incubated for 2 hours with a 1:1,000 dilution a secondary antibody against mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL). Membranes were stripped and reprobed for β-tubulin and optical density values of NOS3 were corrected by β-tubulin. Control experiments for NOS3 Western blot running and transferring method was performed when the technique was optimized. We found that the extreme lanes had lower transference efficiency and that the change in optical density in the 4 central lanes when the same sample was loaded was 0 ± 6 % vs lane #5 (n=3)[88]. Therefore, for all

experiments we only used the central lanes for the experimental samples whereas the remaining lanes were only loaded to ensure even running and transference.

NOS3 pS1177: When phosphorylation of NOS3 at serine 1177 was measured (pS1177 NOS3) THAL suspensions were resuspended in 1 mL. Two aliquots of 300 µL were incubated at 37ºC for 10 min, oxygenating every 5 min. THALs were then treated with vehicle (2.5 µmol/L neomycin neosulfate carrier) or 2.5 µmol/L phosphatidylinositol 3,4,5-triphosphate (PIP3 stock was dissolved in perfusion solution containing Neomycin Neosulfate 1:1 as a carrier, Echelon Biosciences, Salt Lake City, UT) for 8 minutes oxygenating every 4 min. Suspensions were rapidly cooled by adding an equal volume of cold perfusion solution containing 1:50 phosphatase inhibitors (cocktail set II EMD biochemical), spun at 100xg for 2 min and lysed in lysis buffer containing 1:100 phosphatase inhibitors. Western blot for NOS3 pS1177 method was similar to NOS3 but we used 1:2000 primary antibody (BD transduction) [92] and 1:2000 secondary antibody against mouse IgG. Membranes were stripped and reprobed for NOS3 and optical density values for pS1177 were corrected by NOS3.

ETB receptor: Proteins were loaded in 4% stacking- 9% running gels. Membranes were then incubated for 1 hour with blocking buffer at room temperature, followed for 1 hour incubation with a 1:1,250 dilution of anti-ET_B antibody (AER002 Alomone) in blocking buffer, washed 8 times (2 quick, 1x 15 min and 5 x 5') with TBS-T. The membrane was then incubated with a 1:1,000 dilution of secondary antibody against rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL) and washed 10 times (2 quick, 1x 15 min and 7x 5 min). As a control, the primary antibody was pre-incubated with a neutralizing peptide at a peptide/antibody ratio= 1 (Alomone) followed by incubation with a secondary antibody

under the above conditions. The band corresponding to ET_B receptor was absent in the pre-adsorption control experiment. Membranes were stripped and re-probed for β-tubulin and optical densities for ET_B receptor were corrected by β-tubulin.

β-tubulin: Membranes were incubated with stripping buffer (glycine 0.2 mol/L pH 2.8) for 30 min and then washed 4 times with TBS-T. Membranes were then incubated for 1 hour with blocking buffer at room temperature, followed for 1 hour incubation with a 1:10,000 dilution of anti β-tubulin antibody (Abcam, Cambridge, MA) in blocking buffer, washed 5 times with TBS-T and then incubated with a 1:5,000 dilution of a secondary antibody against rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL).

Protein detection and densitometry: The signal was detected by exposure to Fuji Super RX film, which was scanned (EPSON expression 1680 scanner), and densitometry was performed with a custom program. The exposure times for the film were standardized to get bands of mean optical density between 0.40 and 1.00

Measurement of NO production: NO production was measured by fluorescence microscopy in isolated THALs as we have done before[77,82]. In brief, tubules were isolated and transferred to a temperature-regulated chamber (37ºC) and held by two glass pipettes in the absence of flow. The bath was started at 0.5 mL/min and contained 100 µmol/L L-arginine, the substrate for NOS. At this concentration in the absence of flow L-arginine does not stimulate but supports stimulus-induced NO production[90]. Tubules were loaded with the NO-selective fluorescent dye 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF2-FM DA, 4 µmol/L; Molecular Probes, Grand Island, NY) for 20 min, followed by a 20-min wash. Tubules were imaged using a 40x oil immersion objective, and the dye was excited with a xenon lamp

using a 488 nm band pass excitation filter. The fluorescence emitted by NO-bound dye (>500 nm) was measured using Metafluor software (Molecular devices). Measurements were recorded once every 30 sec for a 10-min control period. ET-1 (1 nmol/L prepared in 0.005% acetic acid; Bachem, Torrance, CA), vehicle (0.005% acetic acid), 2.5 µmol/L PIP3 (stock dissolved in perfusion solution containing 1:1 neomycin neosulfate as a carrier, Echelon Biosciences, Salt Lake City, UT) or perfusion solution was then added to the bath. Fluorescence was measured once every 30 sec during a 10-min experimental period. DAF-FM-DA binds NO in an irreversible manner and therefore NO production was measured as the slope of fluorescence over time. Since in the presence of 100 µmol/L L-arginine but not stimuli, NO production is negligible[93], we took the slope of fluorescence over time (Fluorescence units FU/min) obtained during the last 5 min of the basal period as an internal control for dye loading and subtracted it from the highest 5 min slope observed during the experimental period. Of note, the slope of fluorescence over time during the basal period was not different between control and hypertensive rats (for the ET-1 experiments; vehicle: 0.21 ± 0.05 vs Ang: II 0.21 ± 0.06 AU/min; for the PIP3 experiments; vehicle: 0.31 ± 0.08 vs Ang II: 0.27 ± 0.10 AU/min).

Statistical analysis: Results are expressed as mean ± SE. Data were analyzed by the Biostatistics and Research Epidemiology Department of Henry Ford Hospital. Paired t-tests and 2-sample Wilcoxon tests were used as appropriate. When multiple pair-wise comparisons were made, Hochberg's procedure for multiple tests of significance was applied[94].

2. c. Results

First, we measured blood pressure and found that rats infused with 200

ng/Kg/min Ang II for 5 days had a mean arterial blood pressure about 20 mm Hg higher compared with vehicle-treated rats (vehicle: 96 ± 4 mm Hg, n = 5 vs Ang II: 120 ± 8 mm Hg, n = 4; p < 0.02) indicating that this treatment induces a small but significant increase in blood pressure.

Next, we studied whether Ang II-induced hypertension affects NOS3 expression in the THAL. We found that in THALs from Ang II hypertensive rats NOS3 expression was decreased by 40 ± 12% (n = 6, p < 0.007, Figure 2).

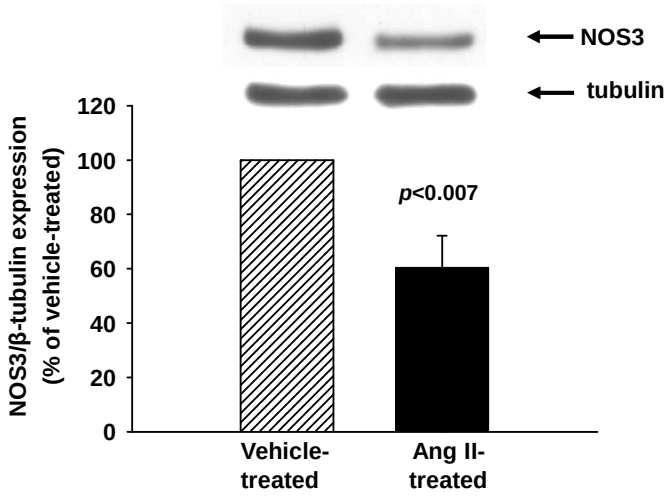

Figure 2. NOS3 protein expression in THALs from vehicle- and Ang II-treated rats. Top: representative Western blot for NOS3. Bottom: cumulative data (n = 6; p < 0.007).

Our lab has shown that ET-1 increases NO *via* NOS3 in the THAL[77]; therefore, we tested whether reduced NOS3 expression resulted in impaired ET-1-induced NO production in Ang II-hypertensive rats. We found that NO production in response to 1 nmol/L ET-1 was increased in THALs from vehicle-treated rats (0.167 ± 0.021 AU/min, n=8) but not in THALs from Ang II-treated rats (-0.012 ± 0.057 AU/min, n=7 p<0.01 Figure 3). These data suggest that reduced NOS3 expression in THALs from Ang II-hypertensive rats correlates with blunted ET-1-induced NO production.

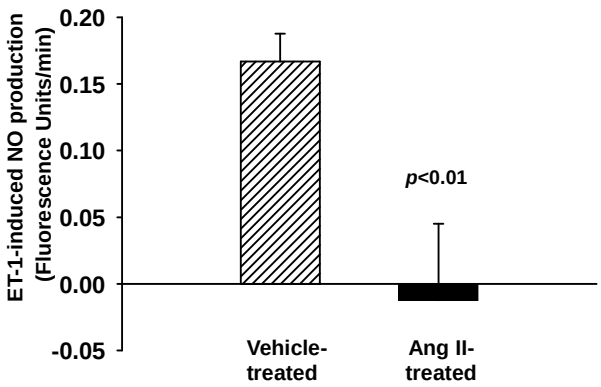

Figure 3. Effect of ET-1 on NO production by isolated THALs from vehicle- and Ang II-treated rats. Intracellular NO production in response to 1 nmol/L ET-1 was measured by fluorescence microscopy in isolated THALs using DAF-FM-DA (vehicle n= 8; Ang II n= 7; $p < 0.01$).

In the THAL ET-1 increases NO production by activating ET_B receptors[87,95]. However, it has been shown that ET_B receptor levels are decreased in renal inner medullas from Ang II-hypertensive rats[46]. To rule out the possibility that impaired NO production in response to ET-1 was due to reduced ET_B receptors, we measured ET_B expression in THALs by Western blot. Contrary to what has been reported in the inner medulla, ET_B receptors were not decreased in THALs from Ang II-hypertensive rats (Δ = 0 ± 15%; n = 6, Figure 4). These data suggest that reduced ET_B receptors are not the likely the cause of impaired ET-1-induced NO production.

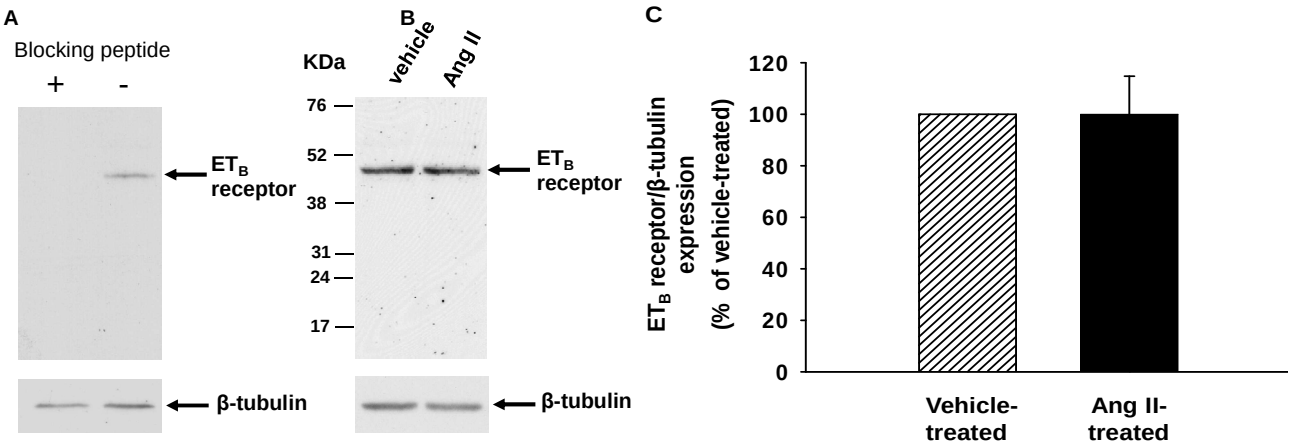

Figure 4. ET_B receptor expression in THALs from vehicle- and Ang II-treated rats. **A)** control Western blot for ET_B showing disappearance of band by pre-incubation of the antibody with a blocking peptide; **B)** representative Western blot for ET_B from THALs from THALs from vehicle- and Ang II-treated rats **C)** cumulative data (n = 6; NS).

In the THAL, activation of NOS3 by hormones and autacoids is mediated by PI3K and its product PIP3[77,89,90]. To test whether impaired ET-1-induced NO production was due to a defect downstream or upstream from PI3K, we measured NO in response to PIP3. PIP3-induced increases in NO were blunted in THALs from Ang II-treated rats (vehicle: 0.128 ± 0.036 *vs* Ang II: -0.065 ± 0.054 AU/min $n = 4$; $p < 0.03$ Figure 5). These data indicate that the defect in NO production in THALs from Ang II hypertensive rats is downstream from PIP3 and therefore likely to be present in response to all the stimuli that utilize this pathway.

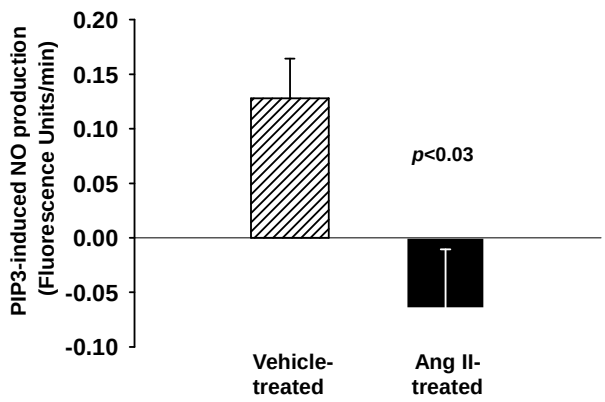

Figure 5. Effect of PIP3 on NO production by isolated THALs from vehicle- and Ang II-treated rats. Intracellular NO production in response to 2.5 µmol/L PIP3 was measured by fluorescence microscopy in isolated THALs using DAF-FM-DA (vehicle n= 4; p < 0.03).

Finally, we tested whether phosphorylation of NOS3 at the stimulatory site serine 1177 in response to PIP3 was also impaired in THALs from Ang II hypertensive rats. Treatment of THALs from vehicle-treated rats with 2.5 µmol/L PIP3 increased pS1177 NOS3/NOS3 by 18 ± 4 % (Figure 6A, n= 5 p< 0.015). However, in THALs from Ang II-hypertensive rats PIP3 was not able to increase phosphorylation at pS1177 (Figure 6B, Δ= - 1 ± 18 %, n= 5). These data indicate that in THALs from Ang II-hypertensive rats stimuli-induced NO production is impaired and this is likely due in part to impaired NOS3 phosphorylation at serine 1177.

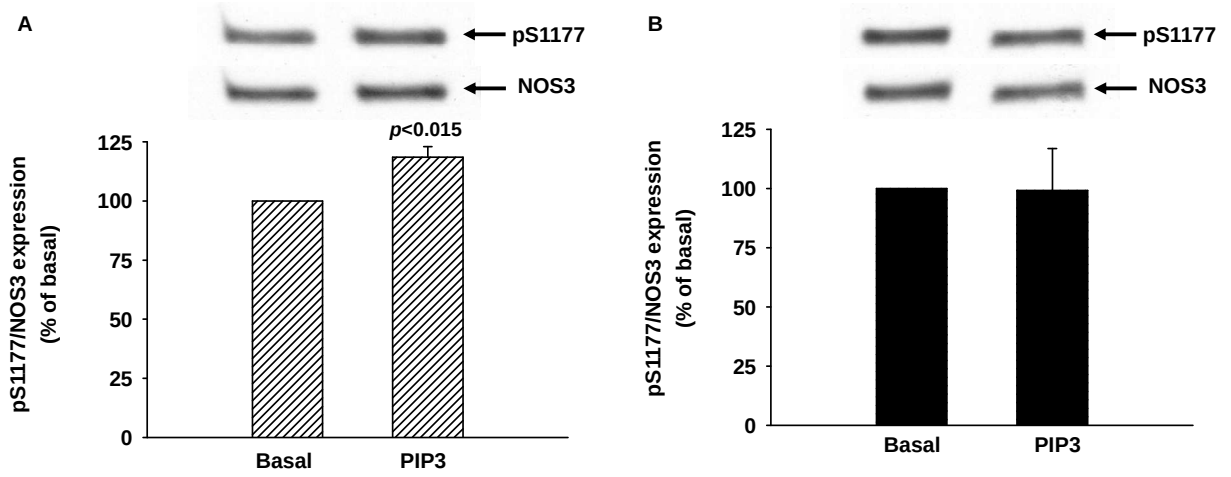

Figure 6. Effect of PIP3 on NOS3 phosphorylation. **A)** Effect of PIP3 on NOS3 phosphorylation at S1177 in THALs from vehicle-treated rats. Cumulative data showing % change in pS1177 NOS3/total NOS3 in response to 2.5 µmol/L PIP3, n= 5. **B)** Effect of PIP3 on NOS3 phosphorylation at S1177 in THALs from Ang II-treated rats. Cumulative data showing % change in pS1177 NOS3/total NOS3 in response to 2.5 µmol/L PIP3, n= 5.

2. d. Discussion

We hypothesized that THAL NOS3 expression is decreased and that NO production in response to physiological stimuli is impaired in Ang II-induced hypertension. We found that in THALs from Ang II-hypertensive rats: 1) NOS3 expression was decreased; 2) ET-1- and PIP3-induced NO production were blunted; 3) ET_B receptor expression was not changed and 4) PIP3-induced phosphorylation of NOS 3 at serine 1177 was increased in THALs from vehicle- but not from Ang II-treated rats. Altogether these data indicate that in Ang II-induced hypertension THAL NO production in response to physiological stimuli is decreased and this is likely due to reduced NOS3 expression and impaired phosphorylation at serine 1177 (Figure 7).

Ang II is a pleiotropic hormone that affects many systems, including the kidneys[96], blood vessels[30], heart[97] and nervous system[98]. Chronically infusing even low

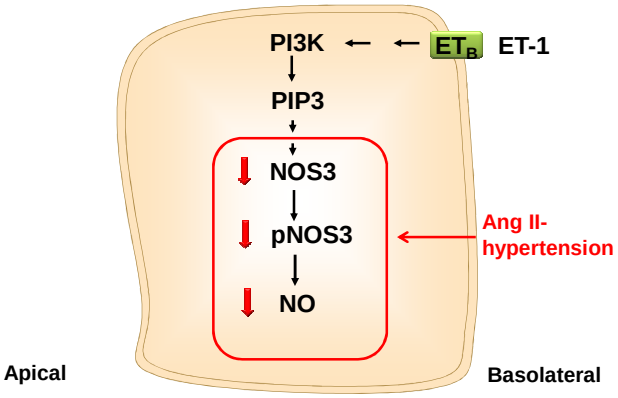

Figure 7. Summary of results from Chapter 2. ET-1 and PIP3 increase NO production by the THAL. Ang II-hypertension impairs this response by reducing THAL NOS3 expression and PIP3-induced NOS3 phosphorylation at serine 1177.

doses of Ang II can shift the pressure natriuresis curve to the right[99,100]. Physiologically this is seen as salt-sensitive hypertension[24,26,27,101,102]. A large range of Ang II dosages has been used to induce hypertension in rats, from 5 ng/kg/min[103] to 600 ng/kg/min[104]; however, at doses exceeding 400 ng/kg/min Ang II-induced hypertension is no longer salt-sensitive[105]. We infused Ang II at a dose of 200 ng/kg/min because at this dose Ang II increases blood pressure in a salt-sensitive manner, indicative of impaired renal function[105].

Most of the studies using Ang II treatment as a way to increase blood pressure, have infused Ang II for 7 days or more[24,39,106,107]. We have chosen 5 days of treatment for several reasons. First, we were interested in studying changes that occur in the THAL in rats exposed to modest yet significant increases in blood pressure. In our study Ang II-hypertensive rats had a mean arterial blood pressure that was about 20 mmHg higher than control rats. Second, we wanted to study how Ang II impairs the ability of physiological stimuli to increase NO production and to inhibit Na reabsorption by the THAL. It has been shown that during the first days of Ang II infusion, Na reabsorption by the kidney is enhanced[24]; however, with the increase in blood pressure, this effect is overcome and increased Na excretion is observed[100]. Furthermore, Crowley et al. showed that cumulative Na excretion measured during the first 5 days of

Ang II infusion was significantly higher in mice lacking AT1 receptors in the kidney compared to WT or AT1 KO mice transplanted with WT kidneys, suggesting that Ang II-induced increases in renal Na reabsorption can be observed during the first 5 days of Ang II infusion and is dependent on effects in the kidney[24]. Finally, we wanted to investigate the causes rather than the consequences of hypertension and exposure to high Ang II levels and high blood pressure for a long period induces renal damage[108,109]. Therefore, we chose 5 days of Ang II treatment to study NO production in response to physiological stimuli as a good compromise between the effect of Ang II in blood pressure, renal Na reabsorption and renal damage.

The THAL expresses all three NOS isoforms: NOS1, 2 and 3[76]. However, unlike the collecting duct, where ET-1 activates NOS1 and NOS3[110-112], in the THAL the NOS isoform that mediates ET-1-induced NO production is NOS3[77]. NOS3 is the source of NO in response to many stimuli including L-arginine[78], ATP[89] and clonidine[79]. Since NOS3 is responsible for stimuli-induced NO production in the THAL, we focused on this isoform. We found that Ang II-induced hypertension decreased NOS3 expression by the THAL.

Although most of the reports in Dahl salt-sensitive hypertensive rats have shown a deficiency in NO production or effects[21,22,71,113,114] the results found in Ang II-hypertension are controversial. It has been shown that infusion of Ang II for 7 days at 600 ng/kg/min[104]; or for 14 days at 280 ng/kg/min did not alter NOS3 expression[75]. In contrast NOS3 expression in the outer medulla was reduced in a model of 2 kidney-1 clip hypertension where Ang II levels are increased[104]. Finally, NO-dependent medullary renal blood flow was reduced in Ang II-hypertensive rats, suggesting that NO levels were reduced in the medulla of Ang II-hypertensive rats[74]. Our results show that

chronic infusion of Ang II reduces NOS3 expression and NO production in the THAL. The reason for the difference between our results and those shown in the whole outer medulla is likely to be the fact that the presence of different type of cells (thin descending limbs, S3 proximal tubules, vasa recta, interstitial cells) may have masked the change observed in THALs. It is also possible that higher increases in blood pressure or longer exposure to hypertension could initiate compensatory mechanisms that increase NOS3. To our knowledge this is the first time that reduced NOS3 expression and NO production by the THAL have been observed in Ang II-induced hypertension.

We have previously shown that ET-1 increases NO production by stimulating NOS3 in the THAL[77]. The NO thus formed inhibits NKCC2 and reduces NaCl reabsorption in this nephron segment[77,87]. Stimulation of NO production by ET-1 is not unique to the THAL. Other cell types like collecting duct cells as well as endothelial cells increase NO production in response to ET-1[110-112]. In those cells concentrations of 0.1 nmol/L to 50 nmol/L ET-1 have been used. We used 1 nmol/L ET-1 based on our previous experience showing that at this concentration ET-1 stimulates NO production and inhibits NKCC2 activity and NaCl reabsorption in THALs[87,95].

Changes in ET_B receptor density or expression in the kidney have been shown in animals fed high salt[115], salt-sensitive spontaneously hypertensive rats[116], and animals treated with Ang II[46,117,118]. In proximal tubules Ang II increases ET_B density by activating AT_1 receptors[119]. In contrast, in inner medullary collecting ducts ET_B density was found to be reduced in heart failure but could be restored to normal by suppressing Ang II production[117]. Kittikulsuth et al[46] recently showed that in Ang II-induced hypertension, ET_B receptor agonist-induced natriuresis was blunted and this correlated

with reduced ET_B receptors in the inner medulla. To test whether ET_B receptor levels were reduced in THALs from Ang II-hypertensive rats we measured ET_B receptor expression. We found that unlike the inner medulla, ET_B receptor expression was not reduced in the THAL. These data suggest that fewer ET_B receptors were not likely to be the cause of the impaired ET-1 response in THALs from hypertensive rats.

Most of the renal natriuretic effects of ET-1 have been attributed to activation of ET_B receptors[120]. ET_A receptors are mainly found in vascular smooth muscle cells and collecting duct cells[120]. To our knowledge neither ET_A receptor expression nor ET_A receptor function has been found in the THAL[87,95,121]. Moreover, inhibition of ET_A receptors did not affect basal NaCl reabsorption, ET-1-induced inhibition of NaCl reabsorption[87] or ET-1-induced increases in NOS3 expression by the THAL[95]. Although we cannot rule out the possibility that Ang II-hypertension induces ET_A receptor expression by the THAL it is not likely to be the cause of impaired ET-1-induced NO production in Ang II-hypertension since the response to PIP3, an ET_B downstream mediator, is also blunted.

Most of the stimuli that increase NO production by the THAL, like ET-1[77], ATP[89], flow[90], Ang II type 2 (AT2) receptor agonists[82] and alpha 2 adrenergic agonists[79], do so by activating PI3K. To study whether the defect observed in Ang II-hypertension was unique to ET-1 or a general phenomenon due to blunted signaling downstream from PI3K, we bypassed the ET_B receptors and stimulated cells with PIP3, the product of PI3K. As expected, PIP3-induced NO production was also blunted in Ang II-hypertensive rats. Altogether, these data suggest that stimuli-induced NO production is impaired in THALs. These data also provides a second line of evidence that reduced ET_B receptor availability is not likely to be the cause of impaired NO production by the

THAL.

NOS3 has at least three putative phosphorylation sites that can alter its activity, but serine 1177 is the key site for stimulation of activity. Phosphorylation at this site has been shown to increase NOS3 activity by enhancing electron flow through the reductase domain and increases NO [122] whereas mutations that prevent this site from being phosphorylated block stimuli-induced NO production [123]. We have shown that in THALs stimuli including ET-1 and flow activate PI3K and increase NOS3 phosphorylation at serine 1177 thus augmenting NO production [77,90]. Multiple kinases like protein kinase B or Akt, protein kinase A, AMP-dependent protein kinase and calmodulin-dependent protein kinase phosphorylate NOS3 at serine 1177 [124,125]. Among them the most studied is Akt. Akt is activated in response to increases in PIP3, the product of PI3K, and in THALs mediates the stimulatory effects of ET-1[77,95], ATP[89] and AT2 receptor agonists on NOS3[82]. In addition, phosphorylation at this site can be reduced by protein phosphatase 2 (PP2)[126,127] and Ang II increases PP2A activity in cardiomyocytes[128]. However, whether Ang II increases PP2 activity in the THAL is not known. We found that phosphorylation of NOS3 at serine 1177 was not increased in response to PIP3 in THALs from Ang II-hypertensive rats, suggesting either reduced Akt activation or enhanced phosphatase activity.

In conclusion, we found that THAL NOS3 expression is decreased and that physiological stimuli-induced NO production and NOS3 phosphorylation at serine 1177 are impaired in Ang II-induced hypertension.

CHAPTER 3

ANGIOTENSIN II DECREASES NOS3 EXPRESSION IN THICK ASCENDING LIMB PRIMARY CULTURES

3. a. Introduction

We have found that Ang II-induced hypertension reduces NOS3 expression in the THAL and impairs the ability of physiological stimuli to increase NO production (Chapter 2). However, whether this is a direct effect of Ang II in the THAL or is secondary to *in vivo* changes observed in Ang II-hypertension (namely increases in blood pressure, elevated lymphocyte infiltration, increases in sympathetic nerve activity) is not clear.

In endothelial cells, increased NO reduces NOS3 expression[80] and activity[81] *via* protein kinase G (PKG) in a negative-feedback fashion. Interestingly acute treatments with Ang II enhanced NO production in the kidney in general[129,130] and in the THAL in particular[82,131-133]. However, whether the same negative feedback is present in THALs in response to Ang II is not known. Therefore, we hypothesized that Ang II decreases NOS3 expression in THALs *via* NO. In this aim we used THAL primary cultures to test the direct effect of Ang II on NOS3 expression and to avoid the confounding effects that increases in blood pressure or chronic Ang II infusion would have on NOS3 expression.

3. b. Materials and Methods

Primary cultures of THALs: All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Hospital and Wayne State University. The composition of physiological saline used was (in mmol/L) 130 NaCl, 2.5 NaH_2PO_4, 4 KCl, 1.2 $MgSO_4$, 6 D/L-alanine, 1 trisodium citrate, 5.5 glucose, 2 calcium dilactate, and 10 HEPES. The solution was adjusted to 320 ± 3

mosmol/kgH_2O with mannitol and was pH 7.4 at room temperature. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained on a diet containing 0.4 % sodium and 1% potassium (Teklad Rodent Diet #8640, Harland-Teklad, Madison, WI). Rats weighing 200–250 g were anesthetized with ketamine and xylazine (100 and 20 mg/kg body wt ip, respectively). THAL suspensions were generated as described in Chapter 2. THALs obtained from two kidneys (one rat) were re-suspended in 1 mL DMEM/F-12 (Invitrogen; Eugene, Oregon) supplemented with 5% heat-inactivated fetal bovine serum (HyClone; Logan, UT), 100 U/mL penicillin, 100 µg/mL streptomycin (HyClone) and 20 ng/mL EGF (Invitrogen; Eugene, Oregon)[95]. Cells were plated on collagen-coated inserts (0.4-µm pore size, 4.7-cm^2 area, Corning Costar, Cambridge, MA) at a concentration of 80 µg protein/insert and placed in an incubator at 37℃ and 95% O_2, 5% CO_2. The use of these inserts promotes cell polarization and allows THALs to be treated on the basolateral and apical side. Previously our lab showed that 92 % of cells in primary cultures were THALs as evidenced by positive Tamm-Horsfall immune-staining[95]. After 40 hrs of seeding, cells were treated with either vehicle (DMEM/F-12 medium) or Ang II 0.1, 1, 10 or 100 nmol/L (Calbiochem; EMD, La Jolla, CA) for 24 hrs. In experiments involving N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, a NOS inhibitor), tempol (a superoxide dismutase mimetic; Sigma St. Louis, MO) or carboxy-PTIO (c-PTIO, a NO scavenger; Cayman Chemical Ann Arbor, Michigan), cells were preincubated for 1 hr with the reagent and then the medium was changed to one containing 100 nmol/L Ang II plus; 1) L-NAME, 2) tempol, 3) c-PTIO or 4) L-NAME + spermine NONOate (NO donor, Cayman Chemical Ann Arbor, Michigan) for 24 hrs. All treatments were added to the basolateral

and apical sides, always included one vehicle (control) and one Ang II (positive control) treatment and were done in the presence of 5% fetal bovine serum.

Western blot: was performed as detailed in Chapter 2.

Statistical analysis: Results are expressed as percentage of control ± standard error. Data were analyzed by the Biostatistics and Research Epidemiology Department from Henry Ford Hospital with an analysis of slope for concentration dependent responses and paired t test. In some experiments ANOVA was used with post hoc testing. When multiple pair-wise comparisons were done, a procedure for multiple tests of significance was applied using Hochberg's significance limits.[94]

3. c. Results

To study the effect of Ang II itself on NOS3 expression and distinguish it from *in vivo* effects, we used THAL primary cultures. Treatment of THALs with 10 and 100 nmol/L Ang II for 24 hrs decreased NOS3 expression by 23 ± 9% (n = 6, $p<0.05$ *vs.* control) and 50 ± 5% (n = 7, $p<0.001$ *vs* control) respectively (Figure 8). Lower Ang II concentrations (0.1 and 1 nmol/L) did not significantly affect NOS3 expression (Δ= -7 ± 10 and Δ= -14 ± 22 *vs.* control, respectively).

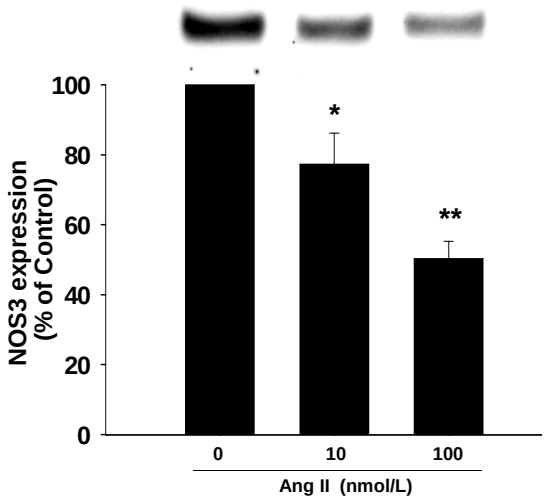

Figure 8. Effect of Ang II in NOS3 expression in THALs**.** THAL primary cultures were incubated with 10 and 100 nmol/L Ang II for 24 hrs and NOS3 expression was assessed by Western blot. Top: Representative Western blot. Bottom: Cumulative data from 6 and 7 independent measurements for 10 and 100nmol/L, *=p<0.05, **=p<0.001 vs 0 nmol/L respectively.

Acutely, Ang II activates NOS in THALs[82,131] and NO negatively regulates NOS3 expression in endothelial cells[80]. Therefore, we next studied whether Ang II decreases NOS3 expression *via* a NO-mediated negative feedback mechanism. First we tested whether NOS activation was required for Ang II-induced reduction of NOS3 expression. In these experiments, 100 nmol/L Ang II alone reduced NOS3 expression by 27 ± 5% (n=5). In contrast, in the presence of L-NAME, a NOS inhibitor, Ang II had no significant effect on NOS3 expression (Δ= -5 ± 8% *vs* control, n=5; *p*<0.007 *vs* Ang II alone; Figure 9). L-NAME alone had no effect on basal NOS3 expression (Δ= -2 ± 8% *vs* control, n=5). These data indicate that NOS activity is required for Ang II to reduce NOS3 expression.

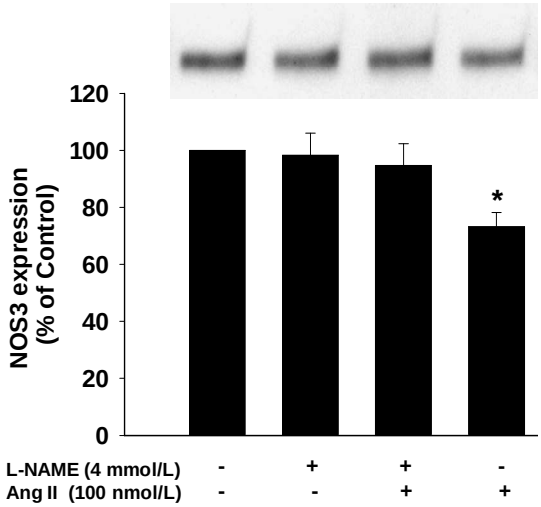

Figure 9. Effect of NOS inhibition on Ang II-induced decrease in NOS3 expression in THALs. Top: Representative Western blot. Bottom: Cumulative data from 5 independent measurements; *=p<0.007 vs L-NAME+ Ang II.

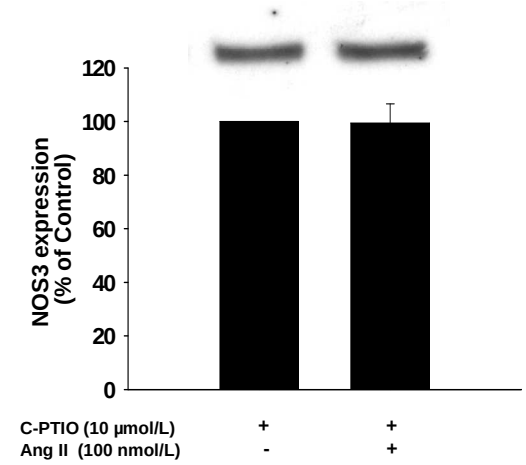

Figure 10. Effect of NO scavenging on Ang II-induced decrease in NOS3 expression in THALs. Top: Representative Western blot. Bottom: Cumulative data from 6 independent measurements, no significant difference.

Next we tested whether NO *per se* was involved in Ang II-induced inhibition of NOS3 expression by using c-PTIO, a NO scavenger[134]. In these experiments Ang II alone reduced NOS3 expression by 33 ± 4% (n = 6). In contrast, in the presence of 10 µmol/L c-PTIO, Ang II had no significant effect on NOS3 expression (Δ= -1 ± 8%, n = 6 *vs* c-PTIO alone; Figure 10). c-PTIO alone did not significantly change NOS3 expression (n=6). Taken together, these data indicate that NO is required for Ang II to reduce NOS3 expression.

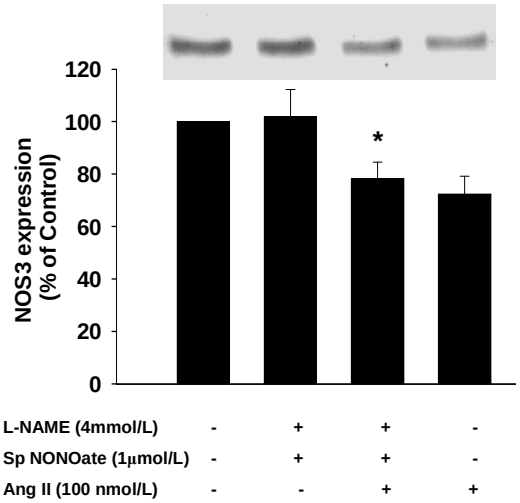

Figure 11. Effect of NOS inhibition and addition of exogenous NO on Ang II-induced decrease in NOS3 expression in THALs. Top: Representative Western blot. Bottom: Cumulative data from 7 independent measurements; *=p<0.013 vs L-NAME + spermine NONOate.

In order to evaluate whether the effect of Ang II on NOS3 expression was only due to NO, we tested the effect of a NO donor on NOS3 expression. To maximize the effect we inhibited endogenous NO production with L-NAME. However, in the presence of L-NAME, addition of the NO donor spermine NONOate (1 µmol/L) did not have any effect on NOS3 expression (Δ= 2 ± 10 %, n = 7). Interestingly, when cells were treated with Ang II in the presence of L-NAME plus the NO donor spermine NONOate (1 µmol/L), NOS3 expression decreased by 22 ± 6% (n=7; *p*<0.013 *vs* L-NAME + NO donor; Figure 11) an effect similar to that observed when Ang II alone was added (Δ= -28 ± 7%, n = 7). These data indicate that NO is necessary but not sufficient to reduce

NOS3 expression in THALs and that simultaneous activation of another mechanism by Ang II is necessary for NO to reduce NOS3 expression.

Ang II not only increases THAL NO but also superoxide production[50,132,133] which reacts with NO to produce the highly oxidant molecule peroxynitrite. Consequently, we studied whether superoxide was also involved in Ang II-induced reductions in NOS3 expression. Ang II alone reduced NOS3 expression by 29 ± 3% (n=6); however, in the presence of tempol, a superoxide scavenger[135], Ang II failed to decrease NOS3 expression (Δ= -3 ± 7%, n = 6; p<0.015 *vs* Ang II) and tempol alone did not affect NOS3 expression (Δ= 6 ± 12%; n = 6; Figure 12).

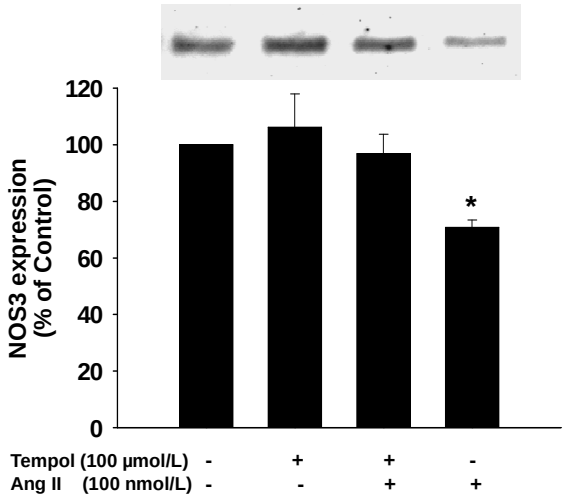

Figure 12. Effect of superoxide dismutation on Ang II-induced decrease in NOS3 expression in THALs. Top: Representative Western blot. Bottom: Cumulative data from 6 independent measurements; *=p<0.015 vs tempol + Ang II.

The tempol results could be due to either a reduction in superoxide which is required for Ang II to decrease NOS3; or the generation of hydrogen peroxide which has been reported to enhance NOS3 expression in endothelial cells[136]. We reasoned that if hydrogen peroxide was masking the effect of NO in NOS3 expression, then in the presence of tempol Ang II should increase NOS3 expression when NOS is inhibited. Thus, we incubated cells with or without Ang II in the presence and absence of both L-NAME and tempol. Ang II alone reduced NOS3 expression by 40 ± 6%. In contrast, Ang

II had no effect on NOS3 expression in the presence of L-NAME and tempol, (Δ= 2 ± 7% *vs* L-NAME+ tempol). L-NAME and tempol did not affect basal NOS3 expression in the absence of Ang II. Taken together, these data indicate that the ability of tempol to block Ang II-induced inhibition of NOS3 expression is due to a reduction in superoxide rather than an increase in hydrogen peroxide.

3. d. Discussion

We found that Ang II decreases NOS3 expression in THALs *via* NO and superoxide and thus is likely mediated by peroxynitrite (Figure 13).

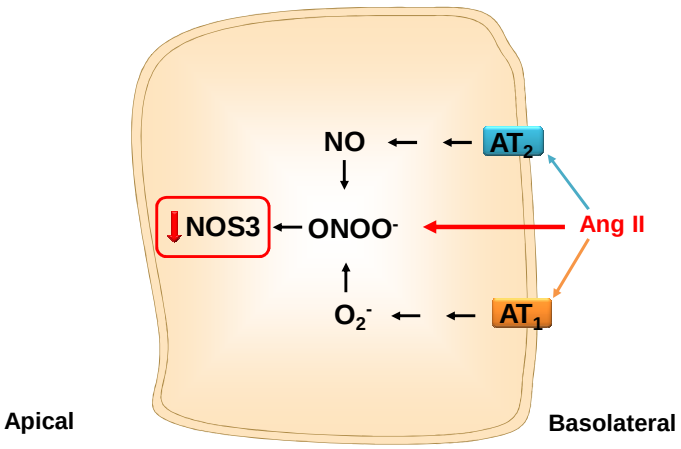

Figure 13. Summary of results from Chapter 3. Acutely, Ang II increases NO *via* AT2 and superoxide (O_2^-) *via* AT1 receptors. Chronic exposure to Ang II results in decreases in THAL NOS3 expression via both NO and superoxide suggesting that peroxynitrite mediates this effect.

We used primary cultures of THALs to avoid confounding influences like those occurring in response to changes in blood pressure[137], renal blood flow[138], tubuloglomerular feedback[139,140], transport in the proximal tubule[141,142], nerve activity[31] or inflammation[143,144] caused by Ang II infusion may have on THAL NOS3 expression. We found that treatment of THAL primary cultures with 10 and 100 nmol/L Ang II for 24 hrs decreased NOS3 expression in a concentration dependent manner. These data are consistent with our previous finding that *in vivo* Ang II reduces NOS3 expression in the THAL. These data also indicate that they are due to a direct effect of Ang II on NOS3 expression rather than secondary to *in vivo* changes induced by chronic Ang II infusion.

In endothelial cells NO donors decrease NOS3 expression [80] and acutely, Ang II increases NO production in the THAL by stimulating the Ang II type 2 (AT2) receptor[82,131]. Thus, we next tested whether Ang II reduces NOS3 expression *via* an increase in NO in THALs. We found that the effect of Ang II on NOS3 expression was blocked by L-NAME indicating that NOS activity was necessary for Ang II to reduce NOS3 expression. Then, we studied whether NO *per se* was involved by scavenging NO with c-PTIO. C-PTIO blocked Ang II-induced decreases in NOS3 expression indicating that NO is indeed required for Ang II to inhibit NOS3 expression in THALs.

One unexpected finding was that in the absence of Ang II, addition of the NO donor spermine NONOate to the incubation medium in the presence of L-NAME did not reduce NOS3 expression, indicating that the effect of Ang II was not solely due to NO and that the simultaneous presence of another signaling molecule was necessary for NO to reduce NOS3 expression. In addition to stimulating NO synthesis, Ang II augments oxidative stress in the THAL by increasing superoxide production[50,54,132,133]; and superoxide in turn, reacts with NO to form peroxynitrite. Thus, we investigated whether superoxide dismutation prevented Ang II from decreasing NOS3 expression. Tempol, a superoxide dismutase mimetic[135], abolished the effect of Ang II on NOS3 expression. These data suggest that superoxide is required for the Ang II-induced decreases in NOS3 expression.

Since superoxide dismutation results in increased hydrogen peroxide [145], and hydrogen peroxide enhances NOS3 expression in endothelial cells [136], the ability of tempol to block Ang II's effect on NOS3 could also be explained by increased hydrogen peroxide. To show that the effect of tempol was due to superoxide dismutation rather than a parallel increase in NOS3 expression induced by hydrogen peroxide, we treated

cells with both L-NAME and tempol. If Ang II increased NOS3 expression in the presence of L-NAME and tempol, this would mean that increased hydrogen peroxide is responsible for the apparent blocking effect of tempol. On the other hand, if Ang II had no effect on NOS3 expression in these experiments, it would indicate that superoxide is required for the Ang II-induced reduction in NOS3 expression. Simultaneous treatment of THAL cells with L-NAME, tempol and Ang II did not change NOS3 expression. Thus we conclude that superoxide participates in Ang II-induced decreases in NOS3 expression.

Our results show that both NO and superoxide are required for Ang II to inhibit NOS3 expression in THALs. Superoxide can react with NO to form peroxynitrite[146] suggesting that peroxynitrite may mediate the actions of Ang II on NOS3. Ang II has been shown to increase tyrosine nitration, a marker of peroxynitrite,[147,148] in endothelial cells[149], proximal tubule[150] and renal outer medulla tissue[151]. In bovine aortic endothelial cells, peroxynitrite decreases NOS3 expression[152] whereas in diabetes an inverse relationship between NOS3 expression and tyrosine nitration has been delineated[153]. Moreover, it has been shown that treatment of endothelial cells with LDL and oxidized LDL results in increased peroxynitrite production and decreased NOS3 expression[154,155] and high glucose reduced NOS3 expression *via* peroxynitrite[133,152,155]. Thus, peroxynitrite may mediate the effects of Ang II on THAL NOS3 expression.

The source of superoxide responsible for Ang II-induced decreases in THAL NOS3 expression needs further study. However, the most likely sources are NADPH oxidase and NOS itself. Ang II increases superoxide production *via* NADPH oxidase in the THAL[133]. Additionally, Ang II induces NOS uncoupling[149,156], thereby increasing superoxide production. Thus, it is possible that NOS itself could become the source of

both the NO and superoxide required for Ang II to inhibit NOS3 expression. Although we showed that L-NAME blocks the effects of Ang II, and L-NAME could block both NO and superoxide production by NOS[156-158] addition of exogenous NO was sufficient to revert the blockade of Ang II-induced decreases in NOS3 caused by L-NAME. Thus, these data appear to rule out the possibility that uncoupled NOS is the source of superoxide.

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CHAPTER 4

NO-MEDIATED INHIBITION OF NKCC2 ACTIVITY IS IMPAIRED IN THICK ASCENDING LIMBS FROM ANGIOTENSIN II-HYPERTENSIVE RATS

4. a. Introduction

NO is an important mediator of natriuresis and vasodilatation. In the THAL endogenous as well as exogenous NO reduces NKCC2 activity[66,77,159]. We have found that THAL NO levels in response to physiological stimuli are impaired in Ang II-induced hypertension (Chapter 2). Similarly, reduced NO production has been shown in salt-sensitive hypertension[113,114] indicating that defects in this signaling mechanism could mediate increases in Na reabsorption and blood pressure. In addition, our lab has shown that NO-induced inhibition of THAL NaCl reabsorption is impaired in THALs from Dahl salt-sensitive hypertensive rats[21,159]. However, whether the response to NO is also impaired in THALs from Ang II-hypertensive rats is not known.

In the THAL NO inhibits NaCl reabsorption by activating soluble guanylate cyclase and increasing cGMP, which reduces NKCC2 levels at the plasma membrane[67,160]. Disruption of NO or cGMP production impairs Na excretion and increases blood pressure[113,161]. cGMP levels can be reduced by cyclic nucleotide PDEs which degrade cGMP by breaking the phosphodiester bond[83]. One important PDE that reduces NO-induced cGMP in smooth muscle cells is PDE5[84,85]. However, whether PDE5 regulates THAL cGMP levels in Ang II-hypertension is not known.

The role of PDE5 in hypertension has been addressed mainly in heart failure and pulmonary hypertension, where PDE5 inhibition is being used as part of the anti-hypertensive therapy[162-164]. Thus most of the studies relating PDE5 and blood pressure have been performed in the vasculature and the heart. However, there has been some

indication of a role of PDE5 in Na reabsorption by the kidney[165-167]. Sasser et al. for example, have shown that PDE5 inhibition increases Na excretion in pregnant versus non-pregnant rats[165]. Interestingly, acute Ang II treatment increases PDE5 and reduces cGMP levels in smooth muscle cells[86] and Ang II-induced hypertension elevates PDE5 expression and activity in cardiomyocytes[168] Therefore, we hypothesized that PDE5 blunts NO-induced increases in cGMP and inhibition of NKCC2 in THALs of Ang II hypertensive rats.

4. b. Methods

Drugs: Unless otherwise stated, reagents and drugs were purchased from Sigma (St. Louis, MO).

Ang II-induced hypertension: As described in Chapter 2.

Isolation and perfusion of rat THALs: THALs were isolated and perfused as it has been done before in our lab[77,159,169,170]. Once the rat was anesthetized, the abdominal cavity was the kidney was bathed in ice-cold saline and removed. Coronal slices were placed in oxygenated physiological saline containing (in mM) 130 NaCl, 2.5 NaH_2PO_4, 4 KCl, 1.2 $MgSO_4$, 6 D/L-alanine, 1 trisodium citrate, 5.5 glucose, 2 calcium dilactate, and 10 HEPES (pH 7.4). THALs were dissected from the outer medulla under a stereomicroscope at 4℃ and transferred to a temperature-regulated chamber, where they were perfused using a concentric glass pipette at 37 ± 1℃ as described previously[77,159].

Measurement of NKCC2 activity: Tubules were perfused at 37℃ in a chamber on the stage of a Nikon Diaphot inverted microscope with a solution designed to prevent Na-K-2Cl cotransporter activity, containing (in mmol/L) 0 Cl, 2.5 NaH_2PO_4, 1.2 $MgSO_4$, 6 L-alanine, 1 Na_3 citrate, 5.5 glucose, 2 Ca dilactate, 10 HEPES (pH 7.45

with KOH) and 237 mannitol. Experiments where ET-1 was used were performed in the presence of 100 µmol/L L-arginine; however, the others were performed in the absence of this aminoacid to avoid endogenous production of NO. THAL cells were loaded by bathing them for 20 min in physiological saline (composition see isolation and perfusion of rat THALs) containing 2 µmol/L Na-sensitive fluorescent dye Sodium Green (Na green Molecular Probes) for measurements of intracellular Na (Na_i). Loading was followed by a 20 min wash with physiological saline. Na green was prepared daily. Na green-loaded tubules were excited using a Xenon arc lamp filtered through two KG1filters and a 488 band pass filter. Emitted fluorescence passed a 505 nm dichroic mirror and then a 515 nm long-pass filter. A cool SNAP HQ2 camera (Photometrics) was used to record the images which were stored and analyzed with a Metafluor system. Images were acquired during 500 msec every 5 sec for a 12 min period. One region covering the whole tubule was used to quantify fluorescence. Then, the luminal solution was switched from 7.4 Na-0Cl-4.8K to 142 Na-134Cl-4K (both containing 100 µM 5-(N,N-dimethyl)amiloride hydrochloride to prevent Na/H exchanger activation). The increase in Na_i caused by the switch is due to activation of the Na-K-2Cl cotransporter[66] and the initial rate of increase in fluorescence was used to calculate cotransport activity. Then the luminal solution was switched back to 7.4 Na-0Cl-4.8K and the tested drug was added to the bath. After 20 min the maneuver was repeated. To calculate NKCC2 activity we measured the slope of fluorescence units(FU) corrected by fluorescence at time 0 (the time at which luminal solutions were switched) times 1000 (represented as arbitrary units or AU) over time in seconds (sec) and we used the first five acquisitions (first 20 seconds) to calculate this initial rate.

When tempol was used to scavenge superoxide, it was added to the bath during

the washing time and kept throughout the experiment (before and after ET-1). NO donor, Spermine NONOate (100 µmol/L, Cayman Chemical Company, Ann Arbor MI) was prepared freshly 2 min before adding to the bath. Dibutyryl c-GMP (db-cGMP) was from ENZO Life Sciences (Ann Arbor, MI). When vardenafil (Bayer AG, Germany) was used to inhibit PDE5, it was added to the bath during the washing time and was kept throughout the experiment (before and after NO).

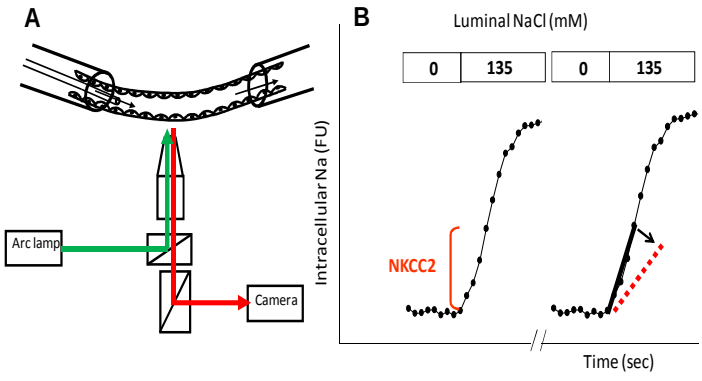


Figure 14. Measurement of NKCC2 activity in isolated THALs by fluorescence microscopy. **A)** Schematic representation of the experimental design. **B)** Type of data collected. NKCC2 activity was calculated from the initial rate of increase in intracellular Na when the luminal solution was exchanged from 0 mmol/L to 130 mmol/NaCl (slope during initial 20 sec period). Arrow exemplifies a hypothetical decrease in NKCC2 activity in the second period (change in slope).

Na green calibration: THALs were isolated, held between two glass pipettes and loaded with Na green as explained in section "Measurement of NKCC2 activity" in the presence of an isosmotic solution containing 3 mM Na in the bath and in the absence of luminal flow. Na green fluorescence was measured once every 30 sec for 500 msec until basal reading was stable. Then the bath was exchanged for one containing 0 mM Na and 400 Units/mL nystatin while acquiring fluorescence measurements. The solution was allowed to equilibrate for 20 min and then exchanged for one containing 3 mM Na. The procedure was repeated with solutions containing 6, 12, 24 and 75 mM Na. All solutions contained 400 Units/mL nystatin, were isosmotic and the concentration of KCl was adjusted for each solution such that [Na+K] = 80 mM.

The last 5 measurements performed with one solution were averaged and used to determine the K_d and Bmax of the dye. We calculated those parameters with the function one site-total binding using the software GraphPad Prism 5. To account for differences in dye loading and compare Bmax from 4 different experiments and obtain one average value, we expressed fluorescence values as fold over fluorescence at 0 mM Na times 1000 (arbitrary units, AU). As expected K_d values were not affected by this conversion and the background signal was 1.

THAL suspensions: Suspensions were performed as explained in Chapter 2.

cGMP measurements: cGMP was measured using a radioimmunoassay kit from Biomedical Technologies (Stoughton, MA) following the manufacturer's instructions. Briefly, THAL suspensions were divided in 4 aliquots. Aliquots 1 and 2 were treated with vehicle (perfusion solution) and aliquots 3 and 4 with vardenafil (25 nmol/L) for 10 min. Then aliquots 1 and 3 were treated with vehicle (perfusion solution) and aliquots 2 and 4 with 100 µmol/L NO donor spermine NONOate (Calbiochem) for 10 min. THALs were lysed with an equal volume of methanol and stored at -80ºC for 2 to 3 hours. Proteins were then spun for 15 min at 15,600g and supernatant* dried overnight in a concentrator (Savant SpeedVac Plus SC110A). The pellet was stored at -20ºC for up to 5 days. Several concentrations of cGMP standards were subjected to the same procedure (dilution in perfusion solution: methanol 1:1 and dried overnight) to evaluate recovery which was 89 ± 11%. The pellet was resuspended in 120 µL assay buffer and acetylated overnight by adding 6µL acetic anhydride: triethylamine at 1:2 ratio. Acetylated samples and standards were assayed following the manufacturer's instructions. Control experiments showed that addition of the non-specific phosphdiesterase inhibitor3-Isobutyl-1-methylxanthine (IBMX increased NO-induced

cGMP by 6 and 17 fold (incubation with NO for 10 and 20 min respectively).

*Proteins precipitated with methanol were resuspended by mechanical disruption of the pellet in perfusion solution, vortexing and sonication (10x1 sec, medium power) and protein concentration measured using a colorimetric assay (Coomassie Plus protein assay, Pierce, Rockford, IL).

PDE5: Western blots were performed as explained in Chapter 2. Membranes were blotted in 5% milk-TBST for 1 hr at room temperature and then incubated for 1 hr with anti-PDE5A1 antibody (BD Transduction, San Jose, CA) 1:750 dilution, washed and incubated with 1:1,000 anti-rabbit antibody (GE Healthcare).

Statistical analysis. Results are expressed as mean ± SE. Data were analyzed by the Biostatistics and Research Epidemiology Department of Henry Ford Hospital. Paired and unpaired t-tests were used as appropriate. When multiple pair-wise comparisons were made, Hochberg's procedure for multiple tests of significance was applied[94].

4. c. Results

First we characterized the method used to measure NKCC2 activity by calculating the K_d of the dye in our conditions and by estimating the changes in intracellular Na that occur during the experimental period. We found that the K_d of the dye was 8.94 ± 2.23 mM Na and that rapid exchange of luminal solution from 0 mM to 134 mM Cl raised intracellular Na from 2.8 to 3.25 mM in 20 seconds (Appendix I).

In normotensive rats, ET-1-induced NO decreases both NKCC2 activity and NaCl reabsorption[77,87] and we have shown that ET-1-induced NO production is impaired in THALs from Ang II-hypertensive rats (Chapter 2). Thus, we first questioned whether this translated into impaired ET-1-induced inhibition of NKCC2 activity in Ang II

hypertension. We found that in vehicle-treated rats ET-1 decreased NKCC2 activity by 38% (from 1.58 ± 0.14 to 0.96 ± 0.16 AU/sec; n= 5; p < 0.04, Figure 15A); however, in hypertensive rats ET-1's ability to decrease NKCC2 activity was blunted (Δ= - 9%, from 1.46 ± 0.24 to 1.23 ± 0.21 AU/sec; n = 6, Figure 15B). These data indicate that the ability of physiological stimuli to inhibit NKCC2 activity is impaired in THALs from Ang II-induced hypertensive rats.

Because superoxide decreases NO bioavailability[171] and enhances NKCC2 activity[51]and our lab has shown that THAL superoxide levels are increased in Ang II-induced hypertension[54], we tested whether the impaired response to ET-1 was due to elevated superoxide. Acutely scavenging superoxide with 100 µmol/L tempol did not

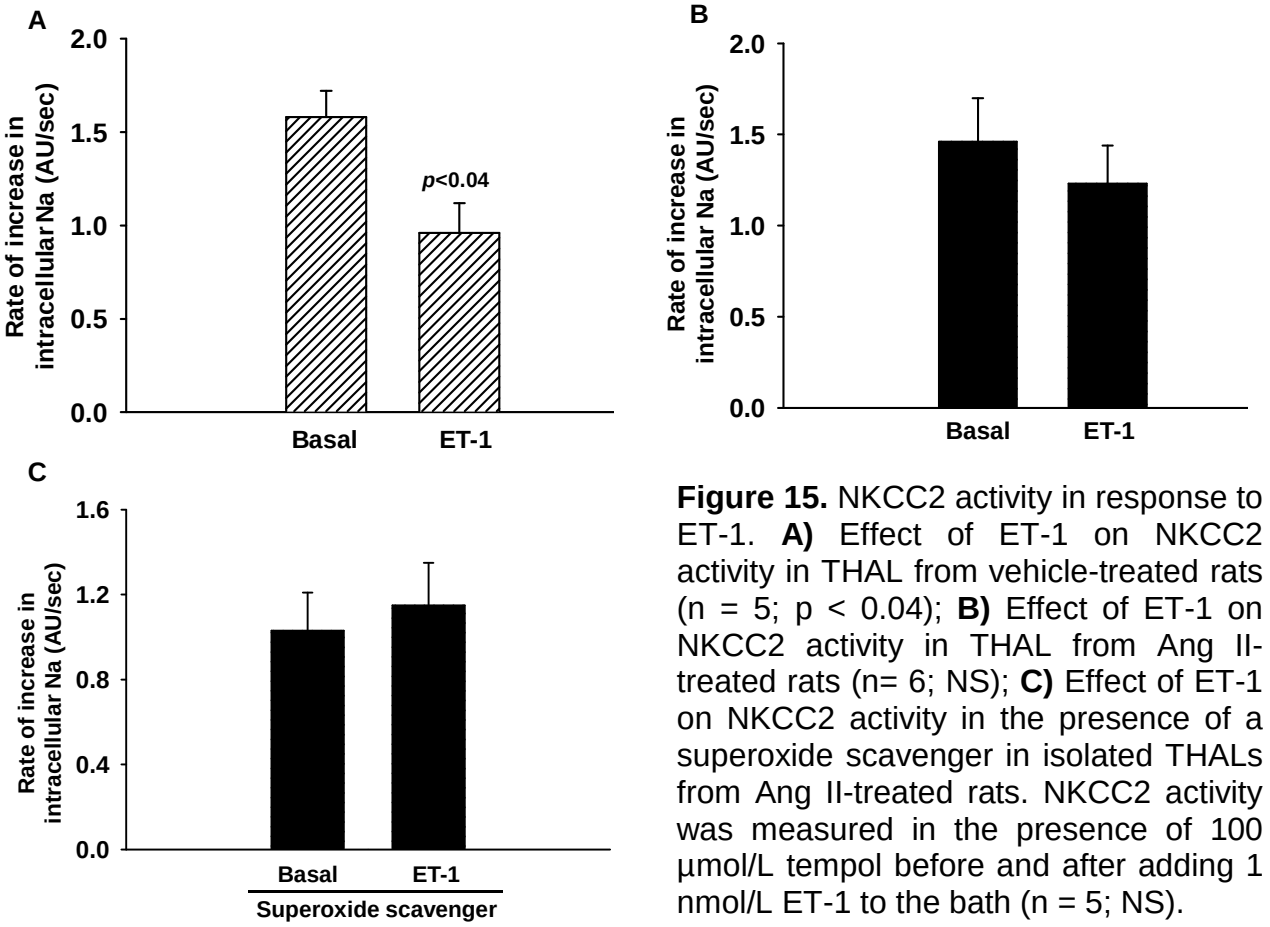


Figure 15. NKCC2 activity in response to ET-1. **A)** Effect of ET-1 on NKCC2 activity in THAL from vehicle-treated rats (n = 5; p < 0.04); **B)** Effect of ET-1 on NKCC2 activity in THAL from Ang II-treated rats (n= 6; NS); **C)** Effect of ET-1 on NKCC2 activity in the presence of a superoxide scavenger in isolated THALs from Ang II-treated rats. NKCC2 activity was measured in the presence of 100 µmol/L tempol before and after adding 1 nmol/L ET-1 to the bath (n = 5; NS).

restore ET-1's ability to decrease NKCC2 in hypertensive rats (tempol: 1.03 ± 0.18 AU/sec; tempol + ET-1: 1.15 ± 0.20 AU/sec, n = 5 Figure 15C). These data indicate that acutely, enhanced superoxide levels in Ang II-treated rats are not responsible for the impaired ability of ET-1 to inhibit NKCC2 activity.

Up to this point our data are consistent with our previous finding that in Ang II-hypertensive rats NO production in response to physiological stimuli is impaired. However, whether NKCC2 response to NO is also affected is not known. Thus, we next measured NKCC2 activity in response to the NO donor spermine NONOate. In THALs from vehicle-treated rats 100 µmol/L spermine NONOate reduced NKCC2 activity by 34%, from 1.68 ± 0.31 to 1.11± 0.31 AU/s ($p<0.01$, n = 6 Figure 16A). In contrast, NO was not able to reduce NKCC2 activity in Ang II-treated rats (basal: 1.13 ± 0.17; NO: 1.32 ± 0.23 AU/s, NS, n = 6, Figure 16B). These data indicate that in addition to reduced NO production, NO-induced inhibition of NKCC2 is blunted in THALs from Ang II-hypertensive rats.

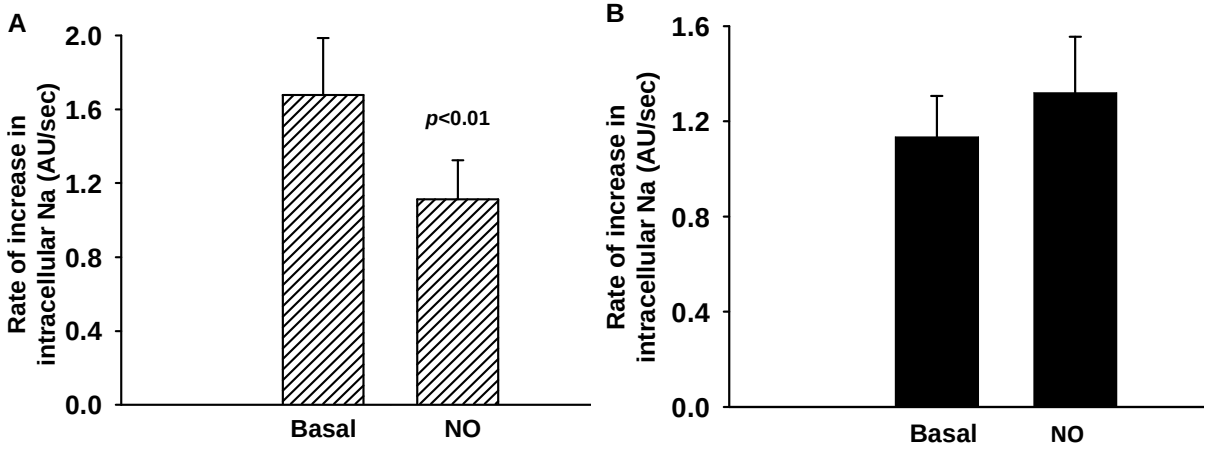


Figure 16. Effect of NO on NKCC2 activity. **A)** Effect of 100 µmol/L spermine NONOate on NKCC2 activity in THALs from vehicle-treated rats (n = 6); **B)** Effect of 100 µmol/L spermine NONOate on NKCC2 activity in THALs from Ang II-treated rats (n = 6).

Impaired NKCC2 inhibition by NO could be due to reduced cGMP production,

increased cGMP degradation or a defect downstream from cGMP. To test whether the response to cGMP was also affected in Ang II-treated rats, we measured NKCC2 activity before and after incubation with 500 µmol/L db-cGMP. In vehicle-treated rats, 500 µmol/L db-cGMP reduced NKCC2 activity by 37% from 1.20 ± 0.32 to 0.76 ± 0.27 ($p<0.02$, n= 5, Figure 17A). Similarly, in Ang II-treated rats 500 µmol/L db-cGMP reduced NKCC2 activity by 41%, from 1.15 ± 0.07 to 0.68 ± 0.10 ($p<0.035$, n = 4, Figure 17B). These data suggest that the signaling cascade downstream from cGMP is intact in Ang II-induced hypertension.

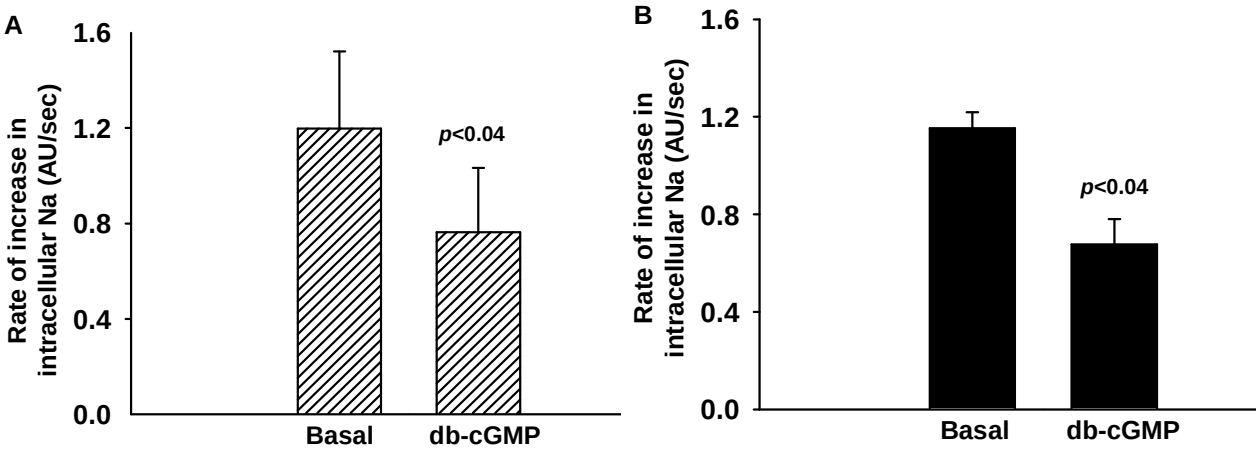
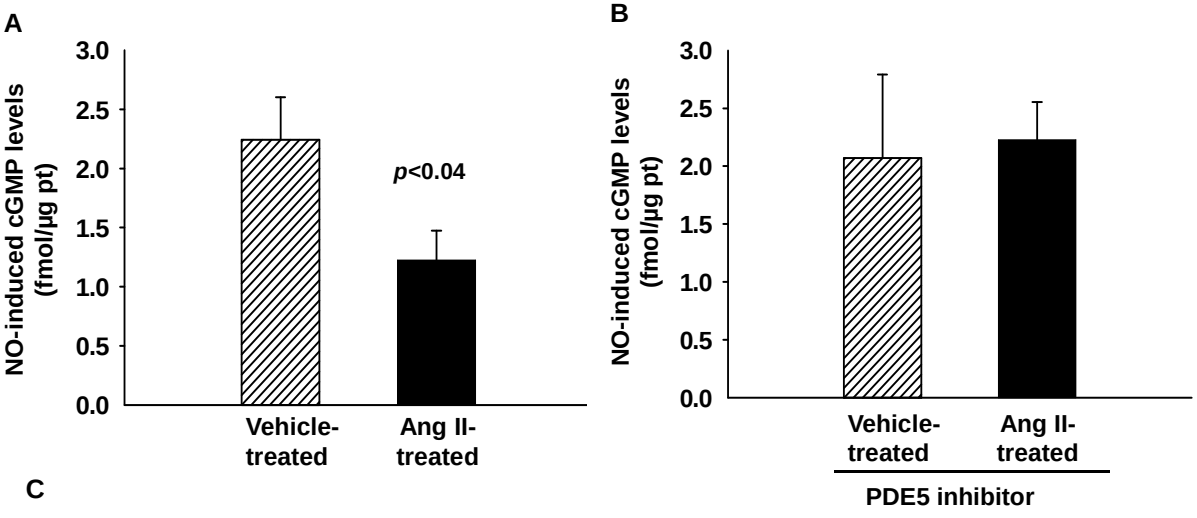


Figure 17. Effect of db-cGMP on NKCC2 activity. **A)** Effect of 500 µmol/L db-cGMP on NKCC2 activity in THALs from vehicle-treated rats (n = 5); **B)** Effect of 500 µmol/L db-cGMP on NKCC2 activity in THALs from Ang II-treated rats (n = 4).

Next, we tested whether the defect was due to an impaired ability of NO to increase cGMP levels. Thus, we measured cGMP in THALs from vehicle and Ang II hypertensive rats. In the absence of PDE inhibition, basal cGMP levels were low, close to the detection level and could only be measured in 3 out of 6 experiments in vehicle-treated rats (0.21 ± 0.03 fmol/µg protein) and in 2 out of 8 experiments in Ang II-treated rats (0.20 fmol/µg protein). Incubation with 100 µmol/L NO increased cGMP more in

THALs from vehicle- than in THALs from Ang II-treated rats (vehicle: 2.24 ± 0.36 fmol/µg pt, n = 6 versus Ang II: 1.22 ± 0.25 fmol/µg pt; n = 8 p<0.04 Figure 18A). These data indicate that the NO-induced increase in cGMP is impaired in THALs from Ang II-hypertensive rats.



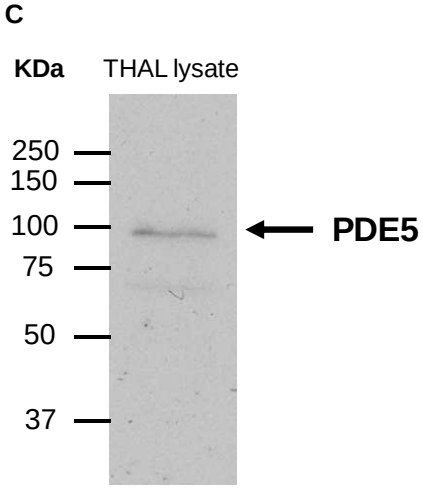


Figure 18. Effect of NO on cGMP levels: role of PDE5. **A)** Effect of 100 µmol/L spermine NONOate on cGMP levels in THALs from vehicle- (n = 6) and Ang II-treated rats (n = 8); **B)** Effect of a PDE5 inhibitor (vardenafil 25 nmol/L) on NO-induced cGMP levels in THALs from vehicle- (n=6) and Ang II-treated rats (n = 8). **C)** PDE5 was detected by Western blot in THAL lysate.

Impaired NO-induced increases in cGMP could be due to reduced production by guanylate cyclase or increased degradation by PDEs. Since it has been shown that *in vitro*, Ang II augments PDE5 activity and cGMP degradation in smooth muscle cells[86] and that *in vivo* Ang II-hypertension enhances PDE5 activity in cardiomyocytes[168], we tested whether inhibition of PDE5 restored NO-induced cGMP in THALs from Ang II-hypertensive rats. First we explored whether PDE5 was present in the THAL. Figure 18C shows a Western blot from THAL lysates depicting a band at the expected

molecular weight of 98 KDa corresponding to PDE5. Next we measured cGMP levels. In the presence of PDE5 inhibition basal cGMP levels were no different between vehicle and Ang II-treated rats (vehicle: 0.21 ± 0.03 fmol/µg protein, n = 3 versus Ang II: 0.25 ± 0.04 fmol/µg protein, n = 4). However, inhibition of PDE5 restored the ability of NO to increase cGMP in THALs from Ang II to the same extent than in vehicle-treated rats (vehicle: 2.07 ± 0.72 fmol/µg protein, n = 6; Ang II: 2.23 ± 0.33 fmol/µg protein, n = 8 NS, Figure 18B). These data indicate that PDE5 blunts NO-induced increases in cGMP in THALs from Ang II-hypertensive rats.

Finally, we tested whether restoration of NO-induced increases in cGMP by PDE5 inhibition translated into restoration of NO-induced reduction in NKCC2 activity in THALs from Ang II-hypertensive rats. In vehicle-treated rats inhibition of PDE5 with 25 nmol/L vardenafil did not affect NO-induced inhibition of NKCC2 (vardenafil: 0.68 ± 0.14; vardenafil + NO: 0.42 ± 0.16 AU/sec, 38% inhibition, p< 0.02, n=4, Figure 19 A).

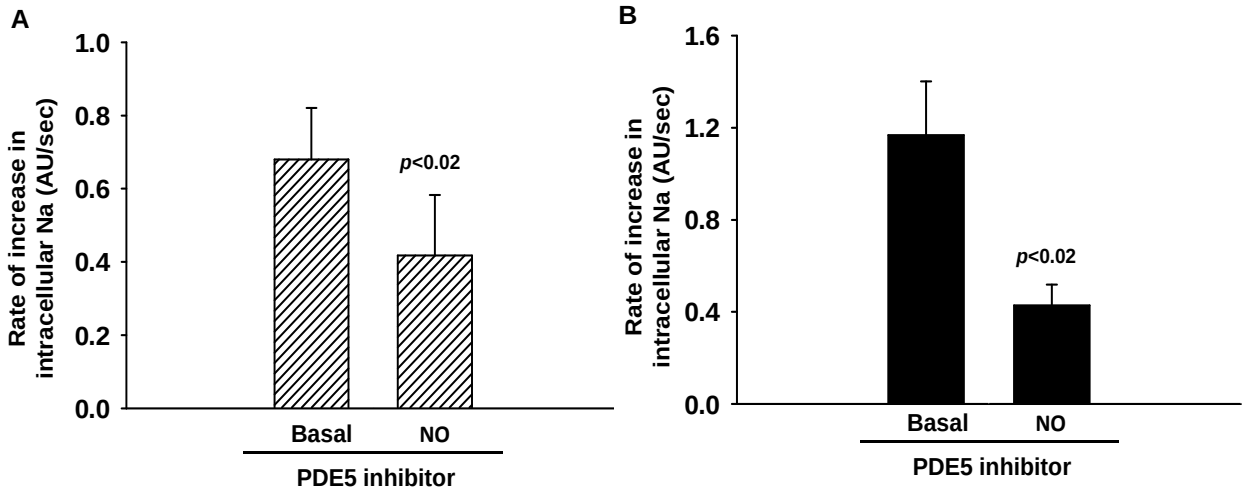


Figure 19. Effect of NO in the presence of a PDE5 inhibitor on NKCC2 activity. **A)** Effect of 100 µmol/L Spermine NONOate in the presence of a PDE5 inhibitor (vardenafil 25 nmol/L) on NKCC2 activity in THALs from vehicle-treated rats (n= 4); **B)** Effect of 100 µmol/L Spermine NONOate in the presence of a PDE5 inhibitor (vardenafil 25 nmol/L) on NKCC2 activity in THALs from Ang II-treated rats (n=5).

However, in Ang II-treated rats, inhibition of PDE5 restored the ability of NO to reduce NKCC2 activity (vardenafil: 1.17 ± 0.23; vardenafil + NO: 0.43 ± 0.1 AU/sec, 63% inhibition, $p<0.02$, n=5, Figure 19B). Time controls with vardenafil showed no difference between first and second period (first period: 0.67 ± 0.15 AU/sec; second period: 0.67 ± 0.03 AU/sec). Altogether these data indicate that PDE5 blunts NO-induced increases in cGMP and inhibition of NKCC2 activity in THALs from Ang II-hypertensive rats.

4. d. Discussion

In this work we found that ET-1-induced inhibition of NKCC2 activity is impaired and that PDE5 blunts NO-induced increases in cGMP and inhibition of NKCC2 activity in THALs from Ang II hypertensive rats (Figure 20). This conclusion is based on the following findings: 1) ET-1-induced inhibition of NKCC2 activity was reduced in THALs from Ang II-hypertensive rats and this was not due to acute increases in superoxide levels; 2) NO-induced inhibition of NKCC2 activity was also impaired in THALs from Ang II-hypertensive rats; 3) db-cGMP inhibited NKCC2 activity to the same extent in Ang II- and vehicle-treated rats; 4) NO-induced increases in cGMP was impaired in THALs from Ang II-hypertensive rats and this was restored by inhibiting PDE5 and 5) NO-induced decreases in NKCC2 activity was restored in THALs from Ang II-hypertensive rats by inhibiting PDE5. To our knowledge this is the first time that impaired NO-induced inhibition of Na reabsorption has been shown in Ang II-induced hypertension. Moreover, this is the first time that PDE5 has been shown to play a role in THAL NaCl reabsorption during Ang II-hypertension.

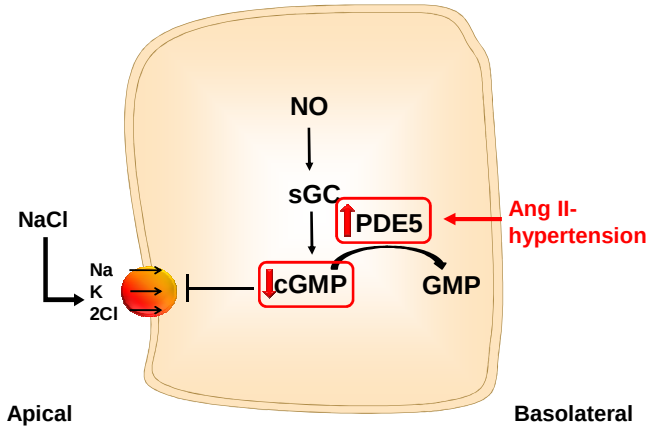


Figure 20. Summary of results from Chapter 4. In THALs NO inhibits NKCC2 activity via cGMP. In Ang II-hypertensive rats, PDE5 blunts NO-induced increases in cGMP and inhibition of NKCC2 activity. sGC=soluble Guanylate cyclase.

ET-1 inhibits NKCC2 by increasing NO, which reduces the number of cotransporters in the luminal membrane *via* cyclic GMP[77,160]. We have found that in Ang II-induced hypertension, physiological stimuli such as ET-1 and PIP3 cannot elicit increases in NO production in THALs and that the defect is related to reductions in NOS3 expression and phosphorylation at the stimulatory site serine 1177 (Chapter 2). However, mechanisms that increase cGMP formation or sensitivity to NO could maintain normal ET-1-induced inhibition of NKCC2. Nevertheless, our findings indicate this is not the case, as impaired ET-1-induced NO production translated to blunted ET-1-induced inhibition of NKCC2.

Since ET-1 is an important regulator of blood pressure and NaCl reabsorption by the kidney and the THAL[120,172], we focused on the ability of this hormone to inhibit NKCC2 activity during hypertension. We found that ET-1-induced inhibition of NKCC2 is impaired in THALs from Ang II hypertensive rats and that this correlates with impaired NO production.

Superoxide levels are increased in many models of hypertension[54,173]. In particular, Ang II acutely and chronically elevates superoxide formation by the THAL[50,54] and superoxide not only reduces NO bioavailability but also directly stimulates NKCC2

activity[51,171,174]. We found that acute superoxide scavenging did not restore the ability of ET-1 to inhibit NKCC2, suggesting that superoxide does not acutely mediate impaired ET-1-induced reduction in NKCC2 activity. This is in contrast to a previous report that showing that chronic infusion of the superoxide scavenger tempol reduced basal NaCl transport by THALs from Ang II-induced hypertensive rats[54]. However, in that study tempol was infused chronically throughout Ang II treatment and basal NaCl reabsorption rather than the response to ET-1 was studied. Interestingly, we have shown that *in vitro* chronic Ang II decreased NOS3 expression in THAL primary cultures and this was prevented by chronic tempol treatment (Chapter 3). Therefore, it is likely that chronic exposure to elevated superoxide mediates the Ang II-induced decrease in NOS3, which ultimately fails to produce NO and inhibit NKCC2 in response to ET-1.

In our lab it was shown that superoxide increases NKCC2 activity[51], that Ang II-hypertension augments superoxide[54] and that scavenging superoxide enhances L-arginine-induced increases in NO production and inhibition of NaCl reabsorption by the THAL[171]. Thus, it is possible that tempol treatment reduced basal NKCC2 activity in THALs from Ang II-hypertensive rats. However, the purpose of our experiment was to test whether the increased superoxide production was responsible for the impaired response to ET-1, either by scavenging ET-1-induced NO or by activating PKC. Our data indicate that acutely, superoxide is not responsible for the defective ET-1-induced-decreases in NKCC2 activity.

Impaired inhibitory response to ET-1 could also be due to reduced response to NO. Our lab has shown that in Dahl salt-sensitive rats, NO-induced inhibition of NaCl reabsorption is decreased[21]. Therefore, we explored whether the signaling cascade downstream from NO was also affected. Similar to what was found in Dahl salt-sensitive

rats, the ability of NO to inhibit NKCC2 activity was impaired in THALs from Ang II hypertensive rats. Resistance to NO donors has also been found in glomeruli of Ang II-hypertensive rats, where cGMP levels in response to sodium nitroprusside was decreased; however, the authors attributed this defect to reduced cGMP production rather than enhanced degradation[175]. Thus to our knowledge this is the first time that impaired response to a NO donor has been shown in any nephron segment in Ang II-induced hypertension.

In order to dissect the mechanism by which Ang II-hypertension blunts the ability of NO to inhibit NKCC2 activity we first studied the response to the cGMP analog db-cGMP. We found that treatment with 500 µmol/L db-cGMP inhibited NKCC2 to the same extent in normotensive and hypertensive rats indicating that the signaling cascade downstream from cGMP is intact in Ang II-treated rats. cGMP inhibits NKCC2 activity in part by stimulating PDE2, which degrades cAMP[160]. Since cAMP levels are important for the maintenance of normal NKCC2 levels at the plasma membrane[176,177], activation of PDE2 decreases cAMP and reduces NKCC2 at the surface. However, our data suggest that these processes are intact in THALs from Ang II-hypertensive rats.

In our experiments, we used 500 µmol/L db-cGMP because at this dose this analog reduces NKCC2 levels at the plasma level by about 45% [160]. Although it seems a high concentration, it has been shown that only about 10% of cyclic nucleotide analogs (with similar permeabilities than db-cGMP) applied extracellularly could be seen inside the cell[178] and thus, intracellular db-cGMP would be about 50 µmol/L. Considering that the Km of PDE2 for cGMP is about 10-31 µmol/L [85,179], we believe that the concentration of db-cGMP we used was appropriate to test the effect of cGMP on NKCC2 activity.

Since the response to cGMP was intact, we next explored whether the defect was due to an inability of THALs from Ang II-treated rats to augment cGMP levels in response to NO. We found that treating THALs with a NO donor increased cGMP levels to a lower extent in THALs from Ang II-hypertensive rats compared with vehicle-treated rats. These data indicate that either cGMP production was reduced or that cGMP degradation was enhanced.

cGMP could be degraded by cGMP-specific PDEs like PDE5, PDE6 and PDE9 or PDEs with dual-specificity like PDE1, 2, 3, 10 and 11 [83]. Since Ang II increases PDE5 expression and activity in smooth muscle cells[86] we focused on PDE5. In order to study whether PDE5 was present in THALs we performed Western blots of THAL lysates. We found a single band at the expected molecular weight of 98 KDa. The presence of PDE5 in the kidney has been shown before but it was found in proximal tubules and collecting ducts[166,180]. Similar to our findings, Dr. Ortiz's lab has shown the presence of PDE5 in THALs (personal communication) confirming our findings that PDE5 is expressed by this nephron segment.

To test whether PDE5 was responsible for reduced cGMP levels in response to NO we treated THALs with the PDE5 inhibitor vardenafil. We found that in the presence of vardenafil, NO stimulated cGMP to the same extent in normotensive and hypertensive rats. Altogether, these data indicate that PDE5 blunts NO-induced increases in cGMP in THALs from Ang II-hypertensive rats. Notably, basal cGMP levels, in the presence or absence of vardenafil, were not different between normotensive and hypertensive rats suggesting that basal PDE5 activity is insignificant and that only after cGMP levels rise, PDE5 activity increases in Ang II-hypertensive rats. In addition, inhibition of PDE5 with 25 nmol/L vardenafil did not affect NO-induced increases in

cGMP levels in vehicle-treated rats indicating that even in the presence of high cGMP levels, PDE5 activity in THALs from normotensive rats remains negligible.

We used vardenafil to inhibit PDE5 because it is very specific and is the most potent PDE5 inhibitor on the market, being more than 10 times more potent than sildenafil and taldalafil[181]. The EC50 for PDE5 is about 0.4 nmol/L[181] and, except for PDE6A and B, is at least 500 times more selective for PDE5 than for any other PDE discovered so far[182]. At the concentration used in this study (25 nmol/L) only PDE5 and PDE6 would be inhibited; however, PDE6 has only been found in the retinal photoreceptors, pineal gland and in some melanoma cells[83] but not in the kidney. Thus, our data strongly indicate that PDE5 is the PDE responsible for enhanced cGMP degradation in THALs from Ang II hypertensive rats.

Finally, we explored whether inhibition of PDE5 would restore the ability of NO to inhibit NKCC2 activity. Blockade of PDE5 with 25 nmol/L vardenafil did not enhance NO-induced inhibition of NKCC2 activity in vehicle-treated rats, but it did restore the ability of NO to reduce NKCC2 activity in Ang II-treated rats. These data indicate that in THALs from Ang II-hypertensive rats PDE5 blunts NO-induced inhibition of NKCC2 activity.

A similar finding has been shown in pregnant rats in which the natriuretic response to a NO donor was only observed in the presence of PDE5 inhibition[165]. Analogous to our results in hypertension, inhibition of PDE5 in inner medullary collecting ducts from pregnant rats also restored the ability of NO donors to increase cGMP levels to the same extent as in non-pregnant rats. However, in contrast to our findings, PDE5 inhibition by itself enhanced Na excretion in pregnant rats. Altogether these data indicate that during pregnancy, NO and cGMP levels in the kidney are increased but

their actions are limited by PDE5 whereas in THALs from Ang II hypertensive rats NO levels are reduced even in response to physiological stimuli and thus enhanced PDE5-mediated degradation of cGMP is only observed in the presence of exogenous NO. Based on our results we can speculate that inhibition of PDE5 alone would not enhance natriuresis in hypertension and thus a combined therapy that simultaneously restores NOS3 activity and inhibits PDE5 in the kidney would be needed to restore the ability of the kidney to properly respond to natriuretic hormones.

In conclusion, in Ang II-induced hypertension PDE5 blunts NO-induced increases in cGMP and inhibition of NKCC2 activity in THALs. The inability of the THAL to respond to this important natriuretic factor likely contributes to the inappropriately enhanced NaCl reabsorption observed in this model of hypertension.

CHAPTER 5

FINAL REMARKS

5. a. Summary

In this work we investigated the response of THALs from Ang II-hypertensive rats to physiological stimuli known to increase NO production and inhibit NKCC2 activity in normotensive rats (Figure 21). We found that in THALs:

1- NO production in response to ET-1- and PIP3 is blunted in Ang II-hypertensive rats and this correlates with reduced NOS3 expression and impaired PIP3-induced increases in NOS3 phosphorylation at serine 1177 (Chapter 2).

2- The effect on NOS3 expression is due to a direct effect of Ang II rather than a consequence of changes occurring *in vivo* (Chapter 3).

3- Ang II-induced reduction in NOS3 expression is mediated by both NO and superoxide and thus is likely due to peroxynitrite (Chapter 3).

4- ET-1-induced inhibition of NKCC2 activity is blunted in Ang II-hypertensive rats and this is not due to acute increases in superoxide production (Chapter 4).

5- NO-induced increases in cGMP and inhibition of NKCC2 activity are also impaired in Ang II-induced hypertension and this can be reversed by PDE5 inhibition (Chapter 4).

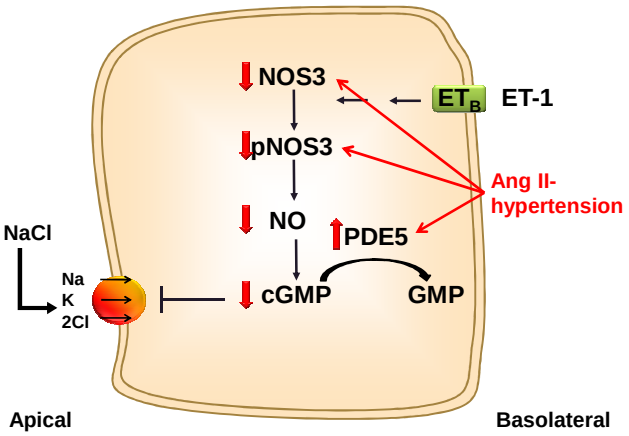


Figure 21. Summary of results. In THALs from normotensive rats ET-1 inhbits NKCC2 activity *via* NO and cGMP. In Ang II-hypertensive rats this response is impaired due to 1) reduced NOS3 expression and phosphorylation at serine 1177, 2) decreased stimuli-induced NO production and 3) enhanced cGMP degradation by PDE5.

5. b. Limitations of the study

Although the methods we used are generally known as being state of the art at this time, we recognize that they have limitations. Here we discuss some of them.

As a general limitation, the use of rodents as a model of human diseases implies that such animals will respond to treatments in the same way that humans do. Although there are high similarities in the physiology of humans and rats, there are many genes that are not expressed in both species and others that have different regulation. However, we believe the use of animals is necessary as a first approach to investigate new signaling mechanisms involved in any pathology.

Another limitation is the age of the rats used. In order to facilitate THAL dissection, we used young rats; however, hypertension usually develops in older subjects and thus the mechanisms involved in the maintenance of high blood pressure could be different.

In this work we studied the response to different treatments of either isolated THALs or THAL suspensions. As with any research performed *in vitro*, there are a number of advantages as well as disadvantages compared to *in vivo* and whole-animal research. Some intrinsic limitations are:

5. b. i. Isolated THALs are studied on a glass chamber outside the renal and body environment, devoid of any interaction with other renal structures, nervous input as well as hormones and autacoids normally present. However, the ability to use isolated and perfused THAL allows us to study THAL function in response to specific stimuli.

5. b. ii. THALs are perfused in a chamber with continuous exchange of the basolateral solution. This maneuver likely washes out some of the autacoids released

by this nephron segment, and thus such responses are missed. However, the basolateral bath in which THALs are kept is continuously exchanged to ensure that appropriate oxygen and nutrient levels are delivered throughout the experiment.

5. b. iii. We studied the response of one stimulus at the time when in reality simultaneous presence of different hormones, metabolites and autacoids likely interact affecting the final result. We did this with the purpose of taking a more practical and easier to analyze approach.

5. b. iv. The osmolality of the basolateral solution was 290 mOsm/L when the osmolality of the outer medulla is about 360 mM[183]. However, given that the osmolality changes with salt-intake and that we wanted to compare our results to those found by others, we used 290 mOsm/L[184-187].

5. b. v. In THAL suspensions, although at low percentage (8%), there is still presence of some cells from other nephron segments. However, at the present moment, this is the best way to study THAL protein expression or production of low-abundance metabolites like cGMP.

5. b. vi. Measurements of NO and NKCC2 activity were done using a single wavelength fluorescent dye. Measurements performed with this type of dye are more susceptible to changes in light intensity, cell volume and dye loading. To avoid some of those problems, we only compared measurements done in the same tubule (not between different tubules), we standardized all our measurements, and we confirmed our findings with other type of experiments (e.g. measurement of NOS3 phosphorylation or cGMP levels). This limitation of the technique also prevented us from studying whether basal NO production and NKCC2 activity were different in THALs from normotensive and hypertensive rats.

5. b. vii. Measurements of NKCC2 activity were done based on the assumption that Na/K ATPase activity was negligible (appendix I); however, such measurements were not made. Therefore, there is a possibility that Na/K ATPase activity could have significantly affected the rate of increase in intracellular Na and that the NKCC2 activity values reported here are lower than the real one.

5. b. viii. Calibration of Na green was done in the presence of nystatin at varying concentrations of Na such that [Na] + [K] = 80 mmol/L and the obtained K_d was about 9 mmol/L. This K_d value is lower than the one previously reported in response to varying concentrations of Na when [Na] + [K] = 135 mol/L. Since K interferes with Na measurement by NaGreen, using a lower than real [K] could lead to an apparent K_d lower than real. However, in contrast to some cells where intracellular K can reach up to 140 mM, in THALs intracellular K has been shown to vary between 80 mmol/L [188] and 117 mmol/L [189]. Furthermore, Despa et al. found that the K_d of NaGreen measured in HeLa cells by lifetime microscopy was 9 mmol/L when using solutions containing [Na]+[K]= 145 mmol/L and that the K_d increased to 21 mmol/L in the presence of albumin [190]. Since our experiments were run in the absence of albumin our values are in agreement with those published in such conditions, and thus we believe it is safe to conclude that the K_d of NaGreen in our system was about 9 mmol/L.

5. b. ix. Stimulation of THALs with ET-1 and PIP3 were done only at one concentration; thus, it is not clear whether there is a shift to the right in the response to these stimuli, in which case higher concentrations would give different results. We chose 1 nmol/L ET-1 based on previous data showing that maximum stimulation of NOS3 and inhibition of THAL NaCl transport in normotensive rats were achieved at this concentration [77,87]. With regard to PIP3 we used a concentration of PIP3 that was 2

orders of magnitude higher than the Km of PIP3 binding to Akt (the enzyme activated by PIP3)[191] to maximally stimulate Akt. Nevertheless, it has been shown that interstitial ET-1 levels in the outer medulla of Ang II-hypertensive rats is not different from control rats suggesting that increases in ET-1 levels do not likely compensate for the impaired response in hypertension. Finally, enhanced PIP3 production in response to ET-1 does not likely play a role since the response to ET-1 was blunted as well.

We studied ET_B receptor expression levels by Western blot. However, ET-1 binding to ET_B receptors could also be affected. ET_B receptor function also depends on its presence at the plasma membrane and on its phosphorylation state[192]. However, we do not believe changes in ET_B receptor function are likely to be the explanation to reduced NO production since stimulation of THALs with PIP3 also failed to increase NO levels.

Although we believe the reduction in NO production was due to decreases in NOS3 expression and NOS3 phosphorylation we cannot exclude the possibility that other factors such as NOS uncoupling, changes in NOS3 protein-protein interactions, NOS3 localization, etc also contribute to impaired NO production. Ideally, restoration of NOS3 expression in the THAL *via* trans-gene expression in Ang II-hypertensive rats would answer the question of whether reductions in NOS3 expression are the only cause of decreases in NO production. In order to know whether impaired phosphorylation of NOS3 in response to PIP3 is the cause of such a defect, we first need to identify the cause of that impairment.

We found that reduced NO production in response to physiological stimuli and increased PDE5-mediated degradation of cGMP were responsible for impaired inhibition of NKCC2 and likely contribute to the elevation in blood pressure in this model

of hypertension. However, we did not test this. THALs are not the only tissue responsible for the elevation of blood pressure in Ang II-hypertension. The THAL's contribution to the increase in blood pressure is likely to be at most 30% (6 mmHg in our model). To extrapolate our results to blood pressure regulation, it would be necessary to perform 1) a THAL-specific inducible-knock down of NOS3 and overactivation of PDE5 and measure blood pressure by telemetry or 2) THAL-specific inducible NOS3 overexpression and PDE5 silencing and study the change in blood pressure in response to Ang II.

5. c. Perspectives

Ang II-induced hypertension has long been used as a model of human hypertension. Although this model has been extensively studied, the mechanisms by which Ang II increases blood pressure have not been completely elucidated.

The role of the kidney in general and the THAL in particular in the regulation of blood pressure is well established, showing that inappropriately elevated NaCl reabsorption is present in hypertension. On one hand, NO produced by NOS3 inhibits THAL NaCl transport and mediates the effect of many natriuretic stimuli. On the other hand, decreases in NO production and impaired response to NO in the renal medulla have been shown in salt-sensitive hypertension. Altogether these data indicate that defects in THAL NO production and actions could also be present in Ang II-hypertension.

In the first part of this work, we showed that chronic Ang II infusion decreases NOS3 expression, impairs NOS3 phosphorylation and reduces stimuli-induced NO production, thus suppressing an important physiological signaling cascade that mediates natriuresis. These findings not only add to previous data showing impaired

natriuresis in hypertension but also propose another defective pathway present in these subjects: reduced THAL NOS3 expression. Decreased NOS3 expression induced by Ang II may be one of the mechanisms by which this hormone enhances Na retention. In addition, these results predict that natriuretic hormones or drugs that depend on NO as a mediator will fail to increase NaCl excretion and imply that some of the beneficial effects of Ang II receptor blockers and angiotensin converting enzyme inhibitors could be due to normalization of NOS3 expression and NO production.

In the second part of our study we explored the signaling mechanism by which Ang II reduces NOS3 expression. In order to directly test the effect of Ang II on NOS3 expression and avoid the confounding effect associated with elevations of Ang II *in vivo* we performed the study in THAL primary cultures. We found that Ang II decreases NOS3 expression in THALs *via* NO and superoxide, raising the possibility that peroxynitrite is involved.

Our lab has previously shown that chronic infusion of the superoxide scavenger tempol into the renal medulla decreases NaCl transport by the THAL in Ang II-hypertensive rats, suggesting that this treatment could restore NOS3 expression and NO production. The effect of Ang II in the THAL is opposite from the findings previously shown in the renal cortex and endothelial cells[193], highlighting the importance of studying the response by each cell type. Understanding the uniqueness of the effect of Ang II in each nephron segment and discerning the pathways that lead to an imbalance between pro and anti-oxidant species would allow for the design of better drugs for the treatment of hypertension.

NO inhibits THAL NaCl reabsorption by inhibiting NKCC2. This cotransporter is not only responsible for all transcellular Cl transport but is instrumental in determining

the lumen-to bath positive potential, which generates the driving force for paracellular cation reabsorption[6,16]. Hormones such as ET-1 stimulate NO production and inhibit NaCl transport primarily by decreasing NKCC2 activity in normotensive rats[77] and elevations in THAL NaCl reabsorption and NKCC2 activity have been found in hypertension[19-21,23,54,194-196]. However, whether regulation of NKCC2 activity by ET-1 is impaired in Ang II-induced hypertension has not been shown before.

In the last part of our work, we found that in addition to reductions in NO production, ET-1-induced inhibition of NKCC2 activity was also blunted in Ang II-hypertension and this was not reversed by acute superoxide scavenging. Moreover, when exploring the response to NO, we found that it was also reduced. NO-induced increases in cGMP as well as NO-induced inhibition of NKCC2 activity were blunted in THALs from Ang II-hypertensive rats and such responses were restored by inhibiting PDE5. Thus, we demonstrated that in THALs from Ang II-induced hypertensive rats PDE5 blunts the ability of NO to increase cGMP and inhibit NKCC2 activity.

The effect of PDE5 in hypertension has been studied before, and PDE5 inhibitors are being used to treat heart failure and pulmonary hypertension[162,163]; however, whether their effectiveness is, at least in part, due to increased Na excretion by the kidney and whether PDE5 inhibitors would reduce blood pressure in other types of hypertension is not clear. Unfortunately, the high expression of PDE5 in other tissues makes this treatment very nonspecific, so discovering the signaling mechanisms that lead to enhanced THAL PDE5 activity during hypertension would be important in designing new drugs for the treatment of high blood pressure.

Finally, if we extrapolate our findings in the THAL to the whole kidney, we would predict that PDE5 inhibitors alone would not be useful in reducing Na retention and

decreasing blood pressure in hypertension. Our data indicate that in Ang II-induced hypertension redundant mechanisms that impair Na excretion by the THAL are present: reduction in NO production in response to physiological stimuli and increased cGMP degradation. Thus, a combination therapy that augments both renal NOS3 expression and activity (like statins and Rho kinase inhibitors) together with PDE5 inhibitors could be effective in enhancing Na excretion and restoring blood pressure to normal values.

APPENDIX I

Calculations of Intracellular Na during Measurements of NKCC2 Activity

In order to validate the method used to measure NKCC2 activity in isolated THALs we calculated the intracellular Na concentration during the measurement. We estimated intracellular Na concentration based on measurements of the dissociation constant of the dye (K_d) and the maximal binding capacity of the dye (Bmax).

I. a. Calculation of intracellular Na

Definitions:

FU_0: Fluorescence at the time when luminal solution is changed from low (0 mM) to high (135 mM) NaCl, first data point in the calculation of the slope of increase in intracellular Na or NKCC2 activity.

AU: Arbitrary units calculated as FU/FU_0*1000.

Approximations:

To calculate K_d and Bmax, total Na was used as a surrogate for free Na. This was done because with the present technology is not possible to separate Na bound to the dye from free Na in an isolated and perfused tubule.

Calculations:

Intracellular Na values were calculated from our data collected in experiments and the calibration of the dye. From calibration experiments we found that Bmax was 0.823 ± 0.017 AU/AU_0 (after subtracting 1.000 ± 0.002 AU/AU_0 for background), K_d was 8.94 ± 2.23 mM Na and non specific binding was 0.003 ± 0.001 AU/AU_0. Since non-specific binding is negligible (less than 3% of the total binding used to estimate Na) we did not include it in our calculations.

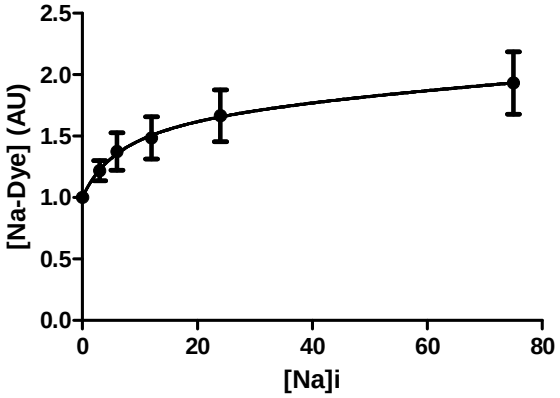


Figure A I. Na green calibration. Cumulative data of 4 experiments showing the change in AU in response to changes in intracellular Na.

Since

$$Na + Dye \leftrightarrow NaDye$$

The constant of dissociation of [NaDye] into [Na] and [Dye] can be represented as

$$Kd = \frac{[Na]f * [Dye]f}{[NaDye]}$$

Where $[Na]_f$ = free intracellular Na
$[Dye]_f$ = free intracellular Dye
[NaDye]= intracellular Na-Dye complex

The intracellular concentration of $[Dye]_f$ is not known; however, we can estimate it from the calibration curve by assuming that at Bmax the maximum available binding sites are bound and thus Bmax could be used as total [Dye].

Considering that

$$[Dye]f = [Dye]t - [NaDye]$$

Then

$$[NaDye] = \frac{[Na]f * ([Dye]t - [NaDye])}{Kd}$$

$$[NaDye] = \frac{[Na]f * Bmax - [Na]f * [NaDye]}{Kd}$$

$$Kd * [NaDye] + [Na]f * [NaDye] = [Na]f * Bmax$$

$$[NaDye] = \frac{[Na]f * Bmax}{Kd + [Na]f} \quad (1)$$

Based on our NKCC2 experiments, increases in intracellular Na when luminal solution changes from 0 mM NaCl to 135 mM Na reaches a plateau and thus likely a steady state condition when fluorescence is 1.30 ± 0.04 AU/AU$_0$, which occurs about 6 minutes after the switch. Thus, based in experiments done in our lab showing that in THALs intracellular Na is 13.5 mM[66] we can calculate theoretical [NaDye] in AU/AU$_0$ units

$$[NaDye] = \frac{0.823\ FU/FU0\ * 13.5\ mM}{8.94\ mM\ +\ 13.5\ mM} = \ 0.495\ AU/AU0$$

then at 13.5 mM Na, Fluorescence should be

$$0.495\ AU/AU0\ +\ 1\ AU/AU0\ of\ background = \ 1.495\ AU/AU0.$$

Since our steady state condition is equal to 1.30 AU/AU$_0$ these data indicate that during THAL perfusion with low Na solution intracellular Na is higher than 0 mM. To calculate this value we substracted 1.30 AU/AU$_0$ (value achieved in plateau during our experiment and expected to be 13.5 mM) to 1.495 AU/AU$_0$ (value that should have been read in the case that the intracellular Na$_0$ was 0 mM).

$$(1.495\ -\ 1.30)\ AU/AU0\ =\ 0.195\ AU/AU0$$

Therefore, we used 0.195 AU/AU$_0$ as a "real" initial value to calculate the concentration of intracellular Na during tubule perfusion with low Na solution. Rearranging equation (1) and calculating:

$$\left(1 - \frac{0.195\ \cancel{AU/AU0}}{0.823\ \cancel{AU/AU0}}\right) * [Na] = \frac{0.195\ \cancel{AU/AU0} * 8.94\ mM}{0.823\ \cancel{AU/AU0}} \qquad (2)$$

$$[Na]\ =\ 2.78\ mM$$

Thus, intracellular Na in the THAL during perfusion with low Na is about 2.78 mM.

Next we questioned what the intracellular Na concentration at the end of the 20 seconds period used to measure NKCC2 activity was. In our experiments, the slope of increase in AU during the 20 sec period varied between 0.9 and 1.68 AU/sec. Thus, the increase in fluorescence from time 0 to 20 seconds varied from 0.018 to 0.033 AU. If we add that to our 0.195 AU "real" value we can calculate intracellular Na after 20 sec perfusion with high Na solution.

Similar to (2)

$$\left(1 - \frac{0.213\ \cancel{AU/AU0}}{0.823\ \cancel{AU/AU0}}\right) * [Na] = \frac{0.213\ \cancel{AU/AU0} * 8.94\ mM}{0.823\ \cancel{AU/AU0}} =$$

$$[Na] = 3.12\ mM$$

$$\left(1 - \frac{0.228\ \cancel{AU/AU0}}{0.823\ \cancel{AU/AU0}}\right) * [Na] = \frac{0.228\ \cancel{AU/AU0} * 8.94\ mM}{0.823\ \cancel{AU/AU0}} =$$

$$[Na] = 3.42\ mM$$

Therefore, at the end of the 20 seconds period intracellular Na would be about 3.1 to 3.4 mM (average 3.25 mM).

Assuming that no post-translational changes occur during this maneuver Na/K ATPase activity can be described by the following model of cation binding described by Garay and Garrahan [197]

$$V = \frac{Vmax}{\frac{1 + KNa''}{[Na]^n}}$$

Where KNa represents the apparent affinity for Na and n represents the number of binding sites, in the case of Na and the Na K ATPase, n=3

Since we do not know KNa, we first calculated that value considering that:

V= ½ Vmax when [Na]= 9.5 mM [198-201]

Therefore KNa

$$\frac{1}{2}Vmax = \frac{Vmax}{\left(1+\frac{KNa}{9.5}\right)^3}$$

$$\sqrt[3]{KNa} = \sqrt[3]{9.5}$$

$$KNa = 2.12\,mM$$

Considering KNa of 2.12 mM we calculated the velocity of the pump at [Na] = 2.78 mM.

$$V = \frac{Vmax}{\left(1+\frac{2.12\cancel{mM}}{2.78\cancel{mM}}\right)^3}$$

$$V = 0.18\,Vmax$$

Calculating the same velocity but using 3.42 mM Na as the highest velocity the pump would have in our measurement we find that

$$V = 0.24\,Vmax$$

Averaging both values to calculate a mean pump velocity during the 20 second period we have

$$V = 0.21\,Vmax\ or\ 21\%\,Vmax$$

Therefore, NaK ATPase activity will vary from 18% to 24% of Vmax during the course of the experiment. However, the ability of the Na/K ATPase to pump Na against its electrochemical gradient depends on the pump electromotive force (Emf) and the electrochemical driving force (V_{DF}) for Na at any given condition. Considering

$$VDF = Vm - VEq$$

where Vm= membrane potential

VEq= equilibrium potential

And THAL basolateral Vm = -65 mV [202]

Therefore, in basal conditions

$$VEq[Na] = \frac{RT}{ZF} * \ln\frac{[Na]o}{[Na]i} = 61.5 \log\frac{135mM}{13mM} = +62.5\ mV$$

and

$$VDF\ (Na) = -65\ mV - 62.5 = 127.5\ mV$$

Since the electromotive force of the Na/K ATPase has been estimated to be between 137 mV[11] and 170 mV [203] at Vm of about -65 mV. These results indicate that in basal conditions (when tubule is perfused with 135 mM NaCl), the electrochemical driving force for Na is lower than the Na/K ATPase pump electromotive force and thus, Na is pumped out of the cell.

However, in conditions where NaCl entry is blocked by perfusing the THAL with a solution containing 0 mM NaCl plus amiloride (Na/H exchanger inhibitor), the VEq[Na] would be:

$$VEq[Na] = 61.5 \log\frac{135mM}{3\ mM} = +102\ mV$$

If we consider that perfusing the THAL with 0 mM NaCl has a similar effect on the basolateral membrane potential as the treatment with furosemide (NKCC2 inhibitor), then basolateral membrane potential would decrease by about 15 mV[199,200] and the V_{DF}[Na] at 0 mM luminal NaCl would be:

$$VDF\ (Na) = -80\ mV - 102 = -182\ mV$$

Although some groups have shown that Na/K ATPase EMF does not depend on Vm [202] others have shown that EMF varies with Vm according to the following equation [203] (the equation considers the change from Vm=0 to the final value in the hyperpolarizing direction):

$$EMF = 134 + 0.56\ \Delta Vm$$

Therefore, at a Vm of -80mV, EMF will have a value between 135 and 178 mV. However, the V_{DF} for Na would be higher than the Na/K ATPase EMF and thus the pump velocity will likely be very low.

I. b. Conclusions

We estimated that during the measurement of NKCC2 activity intracellular Na increased from 2.8 mM after 20 min of perfusion with a solution containing 0 mM NaCl to about 3.4 mM after 20 sec of perfusion with a 130 mM NaCl solution. We also estimated that the Na/K ATPase activity during this maneuver should have been below 21% of maximum activity but likely negligible.

To make these calculations we have used several assumptions and approximations. For example we assumed that total Na could be used as a surrogate of free Na. This approximation would only be correct when Na<<< to Km. However, with the methodology available we cannot separate Na bound to the dye from free Na. This

approach has been widely used since calibrations of fluorescent dyes also base their measurements in this assumption [190,204]. We also assumed that steady-state Nai was 13.5 mM. Although we did not measure intracellular Na in steady state conditions we made this approximation based in previous results from THALs obtained in our lab and therefore we are confident that such values are within the range of intracellular Na present in THALs. Another approximation we made was that the EMF of the pump and the membrane potential was the same as the one recorded in toad bladder. Na/K ATPase EMF has been shown to vary between 135 and 178 mV [202,203] and although some reports show variation with membrane potential others claim that EMF is not affected. Nevertheless it is estimated that at [Na] on the mucosal side of 6 mM or lower Na flux becomes close to 0 in toad bladder [202] suggesting that imposing a steep electrochemical gradient significantly reduces the Na/K ATPase activity to transport Na out of the cell. Although those values can change depending on the conditions used, we believe it is safe to conclude that the Na/K ATP activity will be minimal after 20 min of inhibition of NKCC2 activity by perfusing THALs with 0 mM NaCl based on the following additional evidence: 1) Greger demonstrated that intracellular Na in the presence of NKCC2 inhibition was about 5 mM and that under those conditions Na/K ATPase activity was very low [6]; 2) after NKCC2 inhibition, ouabain does not reduce oxygen comsumption any further suggesting that Na/K ATPase activity in the absence of NKCC2 activity is minimal [205]; 3), in basal conditions ouabain depolarizes THAL cells and induces cell swelling; however, in the presence of furosemide, ouabain does not affect membrane potential or cell volume [6,206] indicating that in the presence of NKCC2 inhibition Na/K ATPase activity is negligible.

Finally, although our estimations indicate that Na/K ATPase activity in the

presence of NKCC2 and NHE3 inhibition would be negligible, there is a possibility that this is not true. If that were the case we expect that the Na/K ATPase activity effect on the measurement of NKCC2 activity is likely to not affect the change in NKCC2 activity observed in response to the acute treatments since we have shown that NO [66] does not affect Na/K ATPase activity in THALs. However, we recognize that in order to ensure this, it would be necessary to measure Na/K ATPase activity in those conditions or to measure changes in intracellular Na in response to ouabain when the THAL is perfused with a solution containing 0 mM NaCl in the presence of dimethyl-amiloride. Absence of any change in intracellular Na would indicate that the Na/K ATPase activity is negligible and thus prove our assumption correct.

In conclusion we believe that measurement of NKCC2 activity monitoring intracellular Na with the fluorescent dye Na green in response to changes in luminal NaCl concentrations is a reliable method to study the activity of this co-transporter in the THAL.

APPENDIX II

Protocol Approval

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 06-15-11

Protocol Effective Period: August 2, 2011 – July 31, 2014

TO: Dr. Vanesa Ramseyer
Physiology
5374 Scott Hall

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 06-15-11

"Effects of Endothelin-1 on Thick Ascending Limb Transport in Angiotensin II-Induced Hypertension (ADMINISTRATIVE]"

DATE: August 2, 2011

Your protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **August 2, 2011** through **July 31, 2014**. The listed source of funding for the protocol is **American Heart Association, Pre-doctoral Fellowship, Midwest Affiliate.** Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active.

The work on the project will not involve the use of live animals conducted within Wayne State University or John D. Dingell VAMC facilities. The institution conducting this work requires an approved IACUC protocol for the live animal work conducted in that facility. However, be aware that the grantee always retains the primary responsibility for ensuring compliance with PHS Policy.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

APPENDIX III

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ABSTRACT

THICK ASCENDING LIMB NITRIC OXIDE PRODUCTION AND INHIBITION OF NKCC2 ACTIVITY ARE IMPAIRED IN ANGIOTENSIN II-INDUCED HYPERTENSION

by

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August 2013

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Major: Physiology

Degree: Doctor of Philosophy

Cardiovascular disease is the leading cause of death in the US. Hypertension, which affects about 30 % of the US population, is an important risk factor in the development of this disease. Inappropriately elevated sodium reabsorption by the kidney contributes to hypertension; therefore, studying the mechanisms that lead to enhanced sodium transport is important in understanding this pathology. The thick ascending limb (THAL) reabsorbs 30% of the filtered sodium chloride load. Nitric oxide (NO) produced by NO synthase type 3 (NOS3) increases cyclic GMP (cGMP) and inhibits THAL transport by reducing Na/K/2Cl cotransporter type 2 (NKCC2) activity. Interestingly, in angiotensin II-induced hypertension, THAL NaCl transport is enhanced. However, whether this is due to a direct effect of angiotensin II on the transporters or to a defect in the NO-signaling pathway is not known. We hypothesized that THAL NO production and inhibition of NKCC2 activity are impaired in angiotensin II-hypertension. We used THALs from normotensive and angiotensin II-infused hypertensive rats. We found that:

1) THAL NO production in response to physiological stimuli was decreased and this correlated with reduced NOS3 expression and phosphorylation at serine 1177 in angiotensin II-induced hypertension;

2) the effect of angiotensin II on NOS3 expression was mediated by peroxynitrite;

3) NO-induced increases in cGMP and inhibition of NKCC2 activity were impaired in angiotensin II-hypertension and this was restored by inhibition of phosphodiesterase 5.

In conclusion, we found that in angiotensin II-induced hypertension THAL NO production is reduced and NO-induced inhibition of NKCC2 activity is blunted due to increased phosphodiesterase 5 activity. We believe this could be one of the mechanisms by which angiotensin II increases NaCl transport by this nephron segment and that treatments that simultaneously increase NO production and inhibit phosphodiesterase 5 activity in the kidney could be useful in the management of hypertension.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

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EXPERIENCE

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HONORS

Scholarships and Fellowships

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2. Graduate Research Assistant Fellowship, Granted by Interdisciplinary Biomedical Sciences Program in Wayne State University, Detroit, MI, period 09/2009-07/2011.
3. Research Fellowship, "Biosynthesis and Thyroid Hormone Action", granted by the National Office of Scientific and Technical Promotion, FONCyT-ANPCyT, Córdoba, Argentina, period 06/2004-06/2005.

Awards (Selected)

1. Hypertension Sydney 2012 special travel award. International Society of Hypertension, Sydney, Australia, October 2012.
2. Pre-doctoral Steven M. Horvath Professional Opportunity Award 2012. American Physiological Society, San Diego, CA, April 2012.
3. Pre-doctoral Research Recognition Award, first place. The American Physiological Society, Water and Electrolyte Homeostasis Section. Experimental Biology, Washington DC, April 2011.
4. Diploma with Honorable Mention graduated with the highest GPA in Masters in Biochemistry, class 2004, granted by the National University of Córdoba, Argentina, year 2002-2003.

Peer-Reviewed Publications

1. **Ramseyer V.D.** and Garvin J.L. Invited Review- Tumor Necrosis Factor alpha: regulation of renal function and blood pressure. American Journal of Physiology, Renal Physiology, 2013 304(10):F1231.
2. **Ramseyer V.D.**, Hong N.J. and Garvin J.L. Tumor Necrosis Factor-α Decreases Nitric Oxide Synthase Type 3 Expression Primarily via Rho/Rho Kinase in the Thick Ascending Limb. Hypertension, 2012; 59(6):1145-50.
3. **Ramseyer V.D.**, Cabral P.D. and Garvin J.L. Book chapter- Role of endothelin in thick ascending limb sodium chloride transport. Contributions to Nephrology, 2011; 172:76-83.
4. **Ramseyer V.D**. and Garvin J.L. Angiotensin II decreases NOS3 expression *via* nitric oxide and superoxide in the thick ascending limb. Hypertension 2009;53[part 2]:313-318.
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